# Concentration-Dependent Effects of Waterborne Zinc on the Interactions between *Gyrodactylus turnbulli* (Monogenea) And the Guppy (*Poecilia reticulata*)

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# ABSTRACT

This research investigated the effects of waterborne zinc (Zn) on the interactions between guppies, *Poecilia reticulata* (Peters), and *Gyrodactylus turnbulli* Harris, 1986, a monogenean parasite of its skin and fins. The first objective was to determine if sublethal concentrations of waterborne Zn (up to 240 µg/L added to artificial freshwater) exerted a concentration-dependent effect on the population dynamics of *Gyrodactylus* on isolated guppies. Whereas survival of uninfected fish was unaffected, mortality of infected fish increased linearly with increasing Zn concentration. In addition, the improved parasite population growth at concentrations up to 120 µg Zn/l suggested either that the elevated Zn promotes survival and/or reproduction of the parasite, or impairs host defense mechanisms. Analysis of lifetime survival and reproduction of individual parasites on and off the fish revealed Zn toxicity to the parasite as survival of detached parasites decreased linearly with increasing Zn concentration and parasite survival on the host was also lower at the highest Zn concentrations. Also, all morphological parameters decreased linearly in response both to increasing concentration and duration of exposure to waterborne Zn. The guppy epidermis responded rapidly to both infection and waterborne Zn, and the cumulative effects of these combined stressors were synergistic for epidermal thickness and mucous cell numbers, but antagonistic in terms of mucin composition. Both Zn and infection induced mucous production, but at elevated Zn concentrations and/or at high parasite burdens, the capacity for continued

mucous production was apparently exceeded. I hypothesize that this condition is favorable for parasite survival because of the impaired host response, but unfavorable for host survival because of the high numbers of pathogenic parasites and the inability to control entry of Zn into host tissues. In conclusion, sublethal concentrations of waterborne Zn are more detrimental to the infected host than to the parasite.

# ABRÉGÉ

Cette thèse a pour but d'étudier l'impact du zinc (Zn) d'origine hydrique sur les interactions entre guppys, *Poecilia reticulata* (Peters), et *Gyrodactylus turnbulli* Harris, 1986, un monogène parasite de la peau et des nageoires. L'objectif premier a été de déterminer si des concentrations sublétales de Zn hydrique (jusqu'à 240 µg/L ajouté en eau douce artificielle) provoquent des effets dépendants de la concentration sur la dynamique de population de *Gyrodactylus* de guppys isolés. Bien que le taux de survie des poissons non infectés soit resté inchangé, la mortalité chez les poissons infectés a augmenté de façon linéaire en fonction des concentrations en Zn. En outre, la croissance de la population du parasite à des concentrations en Zn allant jusqu'à 120 µg/l suggère, d'une part, que la forte présence de Zn favorise la survie ainsi que la reproduction du parasite, et d'autre part, qu'elle compromet les mécanismes de défense de l'hôte. Une étude longitudinale de la survie et de la reproduction de parasites individuels, en présence de l'hôte ou non, indique une toxicité au Zn chez le parasite. Le taux de survie diminue de façon linéaire avec l'augmentation des concentrations en Zn lorsque les parasites ne sont pas rattachés à un hôte, et la mortalité chez les parasites infectants des poissons était aussi plus élevée à de fortes concentrations en Zn. De même, tous les paramètres morphologiques ont diminué de façon linéaire en réponse à l'augmentation des concentrations et de la période d'exposition au Zn hydrique. L'épiderme du guppy a aussi réagi rapidement à l'infection et au Zn hydrique, et l'effet combiné de ces deux agents

de stress est synergique quant à l'épaisseur de l'épiderme et au nombre de cellules muqueuses, mais de nature antagoniste en ce qui concerne la composition de mucines. Le Zn et l'infection induisent la production de mucus à des concentrations élevées en Zn, ainsi qu'à un nombre élevé de parasites; la capacité de production continue de mucus a été apparemment surpassée. Nous pensons que cette condition est favorable à la survie du parasite en raison de la réponse affaiblie de l'hôte, mais défavorable à la survie de l'hôte compte tenu du nombre élevé de parasites pathogènes et à son incapacité de réguler l'entrée du Zn dans ses tissus. En conclusion, des concentrations sublétales de Zn d'origine hydrique sont plus préjudiciables à l'hôte qu'aux parasites.

# STATEMENT OF ORIGINALITY

This was the first study addressing the impact of waterborne Zn on interactions between guppies (*Poecilia reticulata*) and one of their ectoparasites, *Gyrodactylus turnbulli*. The first experiment proved the deleterious effect of waterbone Zn on infected fish. Mortality of the infected fish was significantly higher when compared to the mortality in uninfected fish exposed to the same concentrations of waterborne Zn, and, moreover, increased linearly as the concentration of Zn increased. As the initial results suggested that low to moderate concentrations of Zn could be beneficial to the parasite, we assessed for the first time the effect of waterborne Zn on parasite survival and reproduction of parasites attached to the fish, on the survival of detached parasites and on parasite morphometrics. These results proved the toxic effect of waterborne Zn on parasite survival and morphometrics, and indicate that impaired reproduction in response to Zn may be an indirect consequence of reduced survival. Furthermore, the last set of experiments assessed for the first time the temporal epidermal changes induced by combined waterborne exposure to a range of sublethal concentrations a heavy metal (Zn) together with an ectoparasitic infection (*Gyrodactylus turnbulli*). Previous research has considered infection alone, single concentrations of Zn, a range of concentrations of Zn but at a single time point, or Zn as part of a mix of heavy metals. Present results showed that guppies, recognized to be one of the most resistant fish species to waterborne Zn exposure, mount a rapid epidermal response to waterborne Zn, even in sublethal

concentrations. In addition, combined exposure to Zn and infection were synergistic for epidermal thickness and mucous cells numbers, but antagonistic in terms of mucin composition. These results also explain how Zn concentrations that are toxic to the parasite can promote maximal parasite reproduction because of impaired epithelial responses induced by Zn exposure. Present findings also may have relevance for species used in aquaculture or fisheries that are more susceptible to Zn pollution than guppies and that are also infected with gyrodactylids.

# CONTRIBUTIONS OF AUTHORS

This thesis is written in the form of manuscripts according to the "Guidelines Concerning Thesis Preparation". The thesis consists of three manuscripts co-authored with my supervisor Dr. Scott, Dr. Marcogliese affiliated with St. Lawrence Centre, Environment Canada – Montreal, and with Dr. Cable, affiliated with Cardiff University, Wales, UK. The first two manuscripts have been published, and the third is submitted. Each co-author is mentioned, along with his or her corresponding address, at the beginning of each chapter. Below is a general description of the contribution of each co-author.

I was fortunate to have the rare opportunity to develop a new field of research all the way from conceptualization through to implementation, data processing and interpretation. I was responsible for the experimental design and protocols. In order to be able to complete the experiments, I established a guppy breeding population and a parasite stock culture. Throughout my project, I maintained both the guppy and the parasite stock populations. My responsibilities also included preparation of Zn experimental solutions, all data collection, data analysis and interpretation of the results, as well as the writing of various versions of the manuscripts.

Dr. Scott provided financial resources for the laboratory work and invaluable guidance on the experimental design, data analysis and interpretation of the results. She also provided valuable suggestions for editing the manuscripts that I wrote.

Dr. Marcogliese facilitated my access to St. Lawrence Centre, Environment Canada – Montreal, where I was able to assess Zn concentration of the experimental solutions. He also provided useful background information on the impact of pollutants on wildlife aquatic parasites, and reviewed the final version of the manuscripts that I wrote.

Dr. Cable trained me in parasite handling while I was invited to her lab for a one-week training session. During her subsequent visit to McGill University, she provided important advice and guidance on the choice of the most suitable method for the experiments involving impact of waterborne Zn on attached parasites survival and reproduction, and on the survival of detached parasites, as well as reviewing of the manuscript that I wrote.

# CHAPTER 1

# INTRODUCTION

## 1.1 General introduction

One of our current global concerns is the impact of various pollutants on different ecosystems, including aquatic ones. Fish generally harbor a wide range of parasites which might also be affected by pollution in many ways. Among aquatic parasites, the ectoparasites face a particular challenge, being equally affected by pollutants from the surrounding environment, as well as by the host response, affected in its turn by the pollutants. Moreover, recent reports have shown both increased and decreased parasitism at the polluted sites (Möller, 1987; Khan and Thulin, 1991; Mackenzie *et al.*, 1995).

One important fish ectoparasite is the monogean *Gyrodactylus*, species of which affect almost all species of bony fish in aquaculture, fisheries and hobbyist markets, inducing mortality either directly or through secondary infections (Kearn, 1998; Cone, 1999). These small epidermal feeders attach to their hosts mainly with their posterior opisthaptor and its central pair of hooks and peripheral hooklets (Kearn, 1998). Damage caused by these attachment organs may in turn enhance their hosts' susceptibility to pollutants, as reported for other parasites (Khan and Thulin, 1991).

Environmental pollutants (heavy metals, cyanides, ammonia, polychlorinated biphenyls, pesticides, domestic sewage, pulp and paper effluents, petroleum aromatic hydrocarbons, acid rain, and others) enter the water system

as a consequence of natural watering of the minerals in the earth crust, and of human activities such as mining and metallurgical industries, various pharmaceutical, cosmetics and other industries, agriculture and domestic water wastes. Surprisingly, most studies on the impact of pollutants on aquatic life have focused on the acute toxicity of high concentrations, whereas aquatic organisms (i.e. fish and their parasites) are generally chronically exposed to relatively low, sublethal concentrations of pollutants. Among the heavy metal pollutants of aquatic ecosystems, Zn is the most commonly encountered (Bowen *et al.*, 2006) and has been shown to affect fish in many ways (Atchison *et al.*, 1987; Eisler, 1993; Bowen *et al.*, 2006). Zn has also been reported to affect parasites, especially immature stages of trematodes (Asch and Dresden, 1977; Evans, 1982; Morley *et al.*, 2001a, b; Morley *et al.*, 2002; Morley *et al.*, 2003a, b) and acanthocephalans (Sures, 2002; Thielen *et al.*, 2004).

To date, there are a few studies on the impact of heavy metals on *Gyrodactylus* spp. Most of them used populations of infected fish exposed to a range of sublethal concentrations of heavy metals (especially aluminum (Al) and Zn) and only recorded the impact on parasite and host survival (Soleng *et al.*, 1999; Poléo *et al.*, 2004; Pettersen *et al.*, 2006). There is a lack of studies investigating the mechanisms involved on this complex interactions between heavy metals, parasite and host (e.g. direct toxicity of heavy metals on detached parasites; the impact of toxicants on parasite reproduction and survival; the combined effects of heavy metals and infection on host response).

### 1.2 Objectives

The goals of my research were to document concentration-dependent effects of waterborne zinc on *Gyrodactylus turnbulli* – guppy interactions, and to determine whether they are due to direct effects of Zn on parasite survival or reproduction, or indirect effects mediated through changes in the epidermis. The specific objectives are:

(1) to determine the concentration-dependent effects of Zn on parasite establishment and population growth characteristics and on survival and recovery of infected fish;

(2) to investigate the concentration-dependent effects of Zn on parasite survival, *per capita* rate of parasite reproduction and morphology; and

(3) to document the temporal changes in skin structures induced by Zn alone and/or together with gyrodactylid infection.

### 1.3 The experimental model

As our experimental model we have chosen the guppy (*Poecilia reticulata* (Peters)), and its monogenean parasite, *Gyrodactylus turnbulli*, living on the skin and fins. This host-parasite system is particularly suited to experimental studies because (1) both host and parasite are easy to maintain and breed under laboratory conditions, (2) the parasite has a short generation time (days), and a direct life cycle, without a free-living stage, (3) transmission among hosts occurs as a result of direct fish to fish contact, (4) the infection can be monitored without harming the fish during the experiments (Scott and Anderson, 1984), (5) guppies are useful test animals in aquatic toxicity experiments because they can survive

at very high concentrations of zinc (15 fold of normal body concentration) (Widianarko *et al.*, 2000).

Among the various heavy metal pollutants, Zn was chosen because (1) it is an essential microelement (Watanabe *et al.,* 1997), present in every cell, and involved in the structure or function of more than 300 enzymes and proteins (Vallee and Falchuk, 1993; Cousins, 1998); (2) at elevated concentrations, it becomes an important toxicant (Widianarko *et al.*, 2000, 2001); (3) it is one of the most common aquatic pollutants (Bowen *et al.*, 2006), affecting both fish (Atchison *et al.*, 1987; Eisler, 1993; Bowen *et al.*, 2006) and parasites (Asch and Dresden, 1977; Evans, 1982; Morley *et al.*, 2001a, b; Morley *et al.*, 2002; Sures, 2002; Morley *et al.*, 2003a, b; Thielen *et al.*, 2004) in many ways; (4) the reported toxic concentrations for fish and parasites are within the same order of magnitude whereas cadmium, lead, chromium, mercury and copper are much more toxic for the fish than for aquatic stages of parasites (Galli *et al.*, 1998; Cross *et al.*, 2001; CWQG, 2005; Pietrock *et al.*, 2002; Pietrock and Goater, 2005); and (5) Zn can be used as a model for more toxic metals (e.g. cadmium) (Hogstrand *et al.*, 1994).

**References**


Asch, H.L. and Dresden, M.H., 1977. *Schistosoma mansoni*: effects of zinc on cercarial and schistosomule viability. *J. Parasitol.* 63: 80-86.

Atchison, G.J., Henry, M.G., and Sandheinrich, M.B., 1987. Effects of metals on fish behaviour: a review. *Environ. Biol. Fish.* 18: 11-25.

Bowen, L., Werner, I., and Johnson, M.L., 2006. Physiological and behavioural effects of zinc and temperature on coho salmon (*Oncorhynchus kisutch*). *Hydrobiologia* 559: 161-168.

Cone, D.K., 1999. Monogenea. In *Fish Diseases and Disorders. Vol. 1. Protozoan and Metazoan Infections* (ed. Woo, P.T.K.), pp. 289-327. CABI Publishing, Wallingford, UK.

Cross, M.A., Irwin, S.W. and Fitzpatrick, S.M., 2001. Effects of heavy metal pollution on swimming and longevity in cercariae of Cryptocotyle lingua (Digenea: Heterophyidae). *Parasitology*, 123(5): 499-507.

Eisler, R., 1993. Zinc hazards to fish, wildlife and invertebrates: a synoptic review. U.S. Department of the Interior Fish and Wildlife Service. Patuxent Wildlife Research Center Biological Report 10. Contaminant Hazard Reviews Report 26. Laurel, Maryland 20708.

Evans, N.A., 1982. Effect of copper and zinc upon the survival and infectivity of *Echinoparyphium recurvatum* cercariae. *Parasitology* 85: 295-303.

Galli, P., Crosa, G. and Occhipinti Ambrogi, A., 1998. Heavy metals concentrations in acanthocephalans parasites compared to their fish host. *Chemosphere* 37: 2983-2988.

Hogstrand, C., Wilson R.W., Polgar, D., and Wood, C.M., 1994. Effects of zinc on the kinetics of brachial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. *J.Exp.Biol.* 186: 55-73.

Kearn, G.C., 1998. *Parasitism and the Plathyhelminthes.* (ed. Chapmann and Hall), London, pp. 104-112.

Khan, R.A. and Thulin, J., 1991. Influence of pollution on parasites of aquatic animals. *Adv. Parasitol.* 30: 201-238.

Mackenzie, K., Wiliams, H.H., Wiliams, B., McVicar, A.H., and Siddall, R., 1995. Parasites as indicators of water quality and the potential use of helminth transmission in marine pollution studies. *Adv. Parasitol.* 35: 85-144.

Möller, H., 1987. Pollution and parasitism in the aquatic environment. *Int. J. Parasitology* 17: 353-361.

Morley, N.J., Crane, M. and Lewis, J.W., 2001a. Toxicity of cadmium and zinc to miracidia of *Schistosoma mansoni. Parasitology* 122: 81-85.

Morley, N.J., Crane, M. and Lewis, J.W., 2001b. Toxicity of cadmium and zinc to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. *Int. J. Parasitol.* 31: 1211-1217.

Morley, N.J., Crane, M. and Lewis, J.W., 2002. Toxicity of cadmium and zinc mixtures to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. *Arch. Environ. Contam. Toxicol.*, 43: 28-33.

Morley, N.J., Crane, M., and Lewis, J.W., 2003a. Toxicity of cadmium and zinc to the cercarial activity of *Diplostomum spathaceum* (Trematoda: Diplostomidae). *Folia Parasitologica* 50: 57-60.

Morley, N.J., Crane, M. and Lewis, J.W., 2003b. Effects of cadmium and zinc toxicity on orientation behaviour of *Echinoparyphium recurvatum* (Trematoda: Echinostomidae) cercariae. *Dis. Aquat. Org.* 56: 89-92.

Pettersen, R.A., Vollestad, L.A., Flodmark, L.E. and Poléo, A.B.S., 2006. Effects of aqueous aluminum on four fish ectoparasites. *Sci. Total. Environ.* 369: 129-138.

Pietrock, M. and Goater, C.P., 2005. Infectivity of Ornithodiplostomum ptychocheilus and Posthodiplostomum minimum (Trematoda: Diplostomidae) cercariae following exposure to cadmium. *J. Parasitol.* 91: 854-856.

Pietrock, M., Marcogliese, D., Meinelt, T. and McLaughlin, J., 2002. Effects of mercury and chromium upon longevity of Diplostomum sp. (Trematoda: Diplostomidae) cercariae. *Parasitol. Res.* 3: 225-229.

Poléo, A.B.S., Schjolden, J., Hansen, H., Bakke, T.A., Mo, T.A. and Rosseland, B.O., Lydersen, E., 2004. The effect of various metals on *Gyrodactylus*

*salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology* 128: 169-177.

Scott, M.E. and Anderson, R.M., 1984. The population dynamics of *Gyrodactylus bullatarudis* (Monogenea) within laboratory populations of the fish host *Poecilia reticulata*. *Parasitology* 89: 159-194.

Soleng, A., Poléo, A.B.S., Alstand, N.E.W. and Bakke, T.A., 1999. Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology* 119: 19-25.

Sures, B., 2002. Competition for minerals between *Acanthocephalus lucii* and its definitive host perch (*Perca fluviatilis*) *Int. J. Parasitol.* 32: 1117 – 1122.

Thielen, F., Zimmermann, S., Baska, F., Taraschewski, H. and Sures, B., 2004. The intestinal parasite *Pomphorhynchus laevis* (Acanthocephala) from barbel as a bioindicator for metal pollution in the Danube River near Budapest, Hungary. *Environ. Pollut.* 129: 421-429.

Widianarko, B., van Gestel, C.A.M., Verweij, R.A. and van Straalen, N.M., 2000. Associations between trace metals in sediment, water, and guppy, *Poecilia reticulata* (Peters), from urban streams of Semarag, Indonesia. *Ecotoxicol. Environ. Saf.* 46B: 101-107.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Guppies

Guppies belong to Family Poeciliidae. Their natural range includes northern South America and the adjacent eastern Caribbean islands, but they have been introduced into a number of other tropical countries, either as pets or as mosquito-control agents (Widianarko *et al.*, 2001). Guppies ingest invertebrates, algae, and organic debris (Dussault and Kramer, 1981). Males are smaller (about 3 cm body length excluding tail) but more brightly colored than females (about 6 cm).

The females mature at about 3 months of age and are livebearers, releasing 30 to 60 fry after an average gestation period of 28 days. The males mature earlier, at around 6 weeks. The life span of a well cared-for aquarium guppy is 1.5 to 3 years, depending on genetics and environmental factors (Emmens, 1970). Research conducted on guppies includes taxonomic, genetic, parasitological, toxicological, and behavioral studies, which provide a wide base of information on this fish (i.e. Emmens, 1970; Scott, 1982; Harris, 1988, 1989; Lopez, 1999; Widianarko *et al.*, 2000, 2001; Cable *et al.*, 2002a).

## 2.2 Fish Epithelial Barriers

The fish body surface is in intimate and continuous contact with the aquatic environment. Therefore, fish skin is the first tissue exposed to invading micro-organisms and/or soluble contaminants and the first line of defense. It is a metabolically active tissue that quickly responds to stressors by forming stable physical and/or chemical barriers (Whitear, 1986a; Iger *et al.*, 1994).

Fish skin has two major layers, an outer epidermis and an inner dermis. The epidermis is a stratified epithelium containing epithelial and secretory cells, differing in thickness and number of layers, depending on its location on the body surface, the state of maturity and health of the fish, and the species of fish (Whitear, 1986a). The epidermal cells (also called Malpighian cells or, filament-containing cells, or, more correctly 'epithelial cells') are the predominant cell type. Depending on their location in the epidermis, they are either cuboidal or columnar in the basal layer, or squamous in the superficial layer. It is thought that epithelial cells have mainly structural and protective functions (Schliwa, 1975).

In fish epidermis there are four types of secretory cells (Whitear, 1986a). (1) **Superficial epithelial cells** contain ovoid or spherical secretory vesicles, producing the cuticle or glycocalyx (Schliwa, 1975). (2) **Goblet cells** are exocrine unicellular glands which secrete either a mucoid substance containing glycoproteins - mucous goblet cells (Fletcher *et al.,* 1976), or a proteinaceous secretion - serous goblet cells (Whitear, 1986a). There are regional and temporal differences in the density of goblet cells, and in the production of mucus, depending on the quality of the environment, state of health, and nutritional deficiencies (Roberts and Bullock, 1980). (3) **Sacciform cells** are characterized

by a large vacuole where secretory products accumulate. (4) **Club cells** are large cells in the middle layers of the epidermis, some containing a secretory vacuole. In guppy, epidermis covering the scales in the lateral body sides has an average thickness of 10 μm and consists in 2-3 cell layers, containing mainly epithelial and mucous cells (Schwerdtfeger, 1978). The author reported about 7 mucous cells per mm epidermis length from cross sections of guppy scales.

Both epithelial and secretory cells differentiate from the multipotent progenitor cells, situated usually in the deeper layers, although mitotic activity is present throughout the epidermis (Whitear, 1986a). As new cells appear, the previously differentiated ones are gradually pushed towards the outer layer of the epidermis. In order to become a mature mucous cell, the multipotent progenitor cell differentiates into a precursor mucous cell containing granules of neutral mucins. Later, this precursor cell either shifts the neutral mucopolysaccharide granules into acidic mucopolysaccharide granules and/or synthesizes new acid mucopolysaccharides. In the mature mucous cells, these acidic and neutral polysaccharide granules fuse together forming a complex mixture of both acidic and neutral mucins (Sinha and Chakravorty, 1982).

The epidermis also contains lymphocytes, macrophages and various types of granulocytes (Whitear, 1986a). Among the granulocytes, the most important are epidermal thionin-positive cells (putative mast cells) which are thought to be involved in both specific and non-specific reactions against foreign aggressors. These metachromatically stained cells are located superficially, showing variable densities between fins, with high numbers in the pectoral, pelvic, dorsal and adipose fin, and low numbers on the anal and caudal fin. They are absent in the

cornea. When stained with thionin, the putative mast cells appear deep-blue with red cytoplasmic granules. Sigh and Buchmann (2000) noticed a significant decrease in putative mast cell density during experimental infection of brown trout with either *Ichthyophthirius multifillis* (ciliated protozoan) or *Gyrodactylus derjavini*, suggesting degranulation of these putative mast cells in response to parasitic exposure. Leucocytes are found mainly between and above the basal layer cells, but may be present in any layer of the epidermis, and even at the surface when the skin is damaged. Between the epidermis and dermis, there is a basement membrane, consisting of two layers: an outer *lamina lucida* and an inner *lamina densa* (Whitear, 1986a).

The dermis consists of a connective tissue layer containing mainly collagen (70-80% of dry weight). It provides the substrate and nutrients for maintenance, proliferation and stratification of the epidermis (Sengel, 1986). The dermis also contains chromathophores, blood capillaries, lymphatic vessels and nerves. In addition, the scales of teleosts are developing obliquely in dermal pockets, in which they are anchored by collagenous fibers. Scales consist of collagenous tissue superficially calcified, surrounded by scleroblasts and fibroblasts, and covered by epidermis, despite the fact that in some teleost species, the posterior edges of the scales protrude above the skin surface (Whitear, 1986b).

The inner boundary of the dermis (the dermal endothelium) consists of a thin single sheet of cells. This endothelium has a basement membrane on one or both sides. Below the dermal endothelium lies the hypodermis, which contains deep chromatophores, fat cells, blood vessels and nerves (Whitear, 1986b).

The fish epidermis and its secretions play an important role in protection, in defense against pathogens and pollutants (Whitear, 1986a; Shephard, 1994), in osmoregulation, in locomotion (Whitear, 1986a) and in pheromonal communication (Pickering and Richards, 1980). Normal epithelium is covered by a mucous layer, which is secreted by goblet cells. The mucus and its components have several protective roles against invading pathogens (Shephard, 1994). (1) It prevents the attachment of bacteria, fungi or parasites to epithelial surfaces because it is constantly being sloughed off. (2) If establishment is accomplished, mucus acts as a barrier to be crossed. (3) Finally, the mucus contains a variety of humoral factors with anti-microbial proprieties: lysosyme, complement, lectins, and proteolytic enzymes (Buchmann and Bresciani, 1998). (4) Fish are able to synthesize secretory antibodies at the mucous surface, independently of systemic production of immunoglobulins (St. Louis-Cormier *et al.*, 1984). In addition, Rombout *et al.* (1993) reported structural and functional differences between serum and local immunoglobulins.

Mucus is also extremely important for the protection against environmental pollutants including heavy metals. Several studies have shown that Zn stimulates mucus secretion in fish (Iger *et al.*, 1994; Khunyakari *et al.*, 2001). This increase in mucus secretion is supposed to protect fish from metal pollutants. At a neutral pH, mucus is polyanionic, having a large metal binding capacity (Handy *et al.*, 1989). Shephard (1994) suggested that mucus is also involved in the binding and precipitation of potential toxicants, preventing them from reaching other uptake surfaces, thus regulating Zn absorption. As a consequence, the mucus and epithelial cells accumulated up to 74% of the

retained $Zn^{2+}$, which greatly reduced exposure of underlining tissues to this potential toxicant. Also, the continual sloughing of both mucus and epithelial cells excluded the majority of Zn from absorption (Handy *et al.*, 1989).

### 2.3 Gyrodactylus

*Gyrodactylus* species are ectoparasites belonging to the phylum Platyhelminthes (Flatworms), class Monogenea, family Gyrodactylidae, genus *Gyrodactylus*, which live on the skin, fins, and gills of many families of marine and freshwater teleost fish (Cable *et al.*, 1998; Reed *et al.*, 1998; Cone, 1999). These recognized pathogens in aquaculture and in the aquarium hobbyist market cause mortality directly or indirectly through secondary bacterial or fungal infection (Cone, 1999). The most economically important species is *Gyrodactylus salaris*, a significant pathogen of wild Atlantic salmon in Norway (Johnsen and Jensen, 1991; Bakke *et al.*, 1992; 2000). Atlantic salmon (*Salmo salar*) is the most susceptible species and is unable to control parasite population growth, whereas Baltic salmon (Bakke *et al.*, 1990) and other salmonids (Bakke *et al.*, 1996) can limit infections (Harris *et al.*, 1998).

There are over 400 species of *Gyrodactylus*, with an extremely conservative morphology (Cable and Harris, 2002). They attach themselves to the host by two adhesive organs situated at the extremities of the body. The main adhesive organ (the opisthaptor), provides virtually permanent attachment to the host, and is located at the posterior end. The opisthaptor has one central pair of large hooks (hamuli), and 16 marginal hooklets (Kearn, 1998). The anterior adhesive organs are ventrally directed and used for brief attachment during locomotion, helping the parasite maintain contact with the host while the opisthaptor is detached. Systematics of the group is based on: (1) size and shape of the hard parts of the opisthaptor (Malmberg, 1990; Shinn *et al.*, 1993); (2) organization of the excretory system (Malmberg, 1990), (3) distribution of

tegumental sensilla (Shinn *et al.*, 1997), and, more recently, (4) conserved ribosomal DNA sequences (Cunningham, 1997).

Gyrodactylids are unique within the Animal Kingdom, due to their extraordinary reproductive adaptations (Harris, 1989). *Gyrodactylus* individuals are females at birth, then hermaphroditic, as the male system develops only after the parasite has given birth for the first time (Harris, 1985). They are also viviparous. The young newborn (fully-developed offspring) resembles a 'Russian doll', containing within the uterus several generations of embryos in sequential stages of development (paedogenesis) (Cable *et al.*, 1998). Of particular interest is the development of embryos. The enormous uterus contains an embryo (F1), which in its turn contains another embryo, (F2), which contains another embryo (F3). Moreover, under the uterus there is an egg (Fig.2.1).

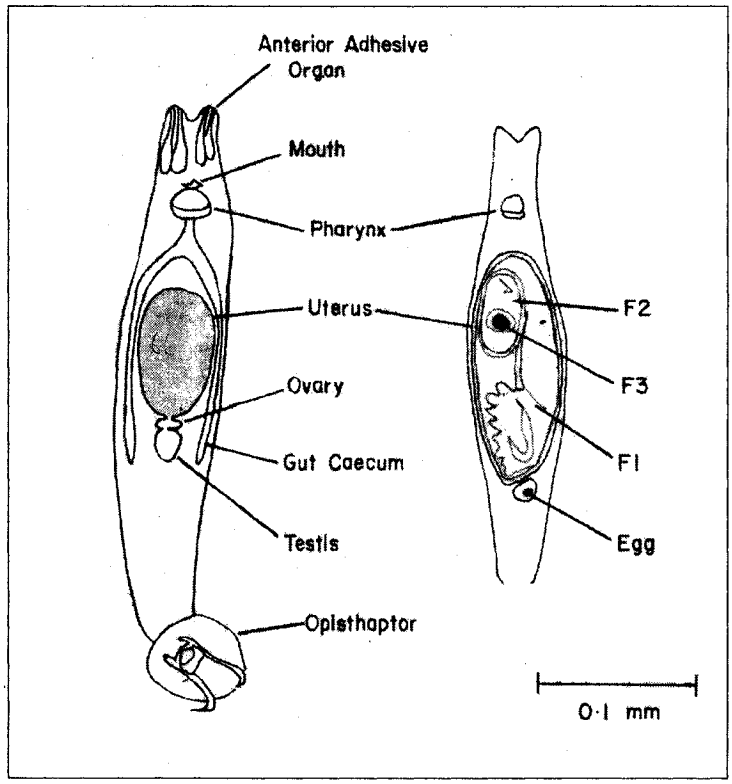


Fig. 2.1 *Gyrodactylus* morphology

(reprinted with permission of Dr. Marilyn E. Scott)

*Gyrodactylus* has a direct life cycle (Fig.2.2). The first generation derives from an embryo cluster *in utero*, as an asexual clone of its mother, whereas subsequent ones derive from oocytes, being either sexually or parthenogenetically-formed (Harris, 1989; Harris *et al.*, 1994; Cable and Harris, 2002). However, the newly born parasites are morphologically identical, and attach immediately to the host, near their mother (Scott, 1982). As a result, *Gyrodactylus turnbulli* on guppies can produce at least nine consecutive generations (Scott, 1982) in the absence of cross-fertilization, at daily intervals, without reducing the average birth rate for each generation (Scott, 1982). Mating can take place between later stages. According to Harris (1989), the population dynamics of *G. turnbulli* is influenced by sexual reproduction, as there are periods of small parasite populations when multiplication is predominantly asexual alternating with phases of large and crowded populations favoring cross-insemination.

Gyrodactylids lack the usual dispersal stage of other monogeneans – the oncomiracidium. To assure their dispersal, gyrodactylids are mobile and able to change microhabitats. *G. salaris* transfers between hosts through direct contact with live and/or dead hosts, or through detached or attached parasites to the substrate, all these being temperature-dependent (Bakke *et al.*, 1992; Soleng *et al.*, 1999). The most important form of dispersal from infected to uninfected host is during direct contact with an infected fish (Scott and Anderson, 1984; Soleng *et al.*, 1999).

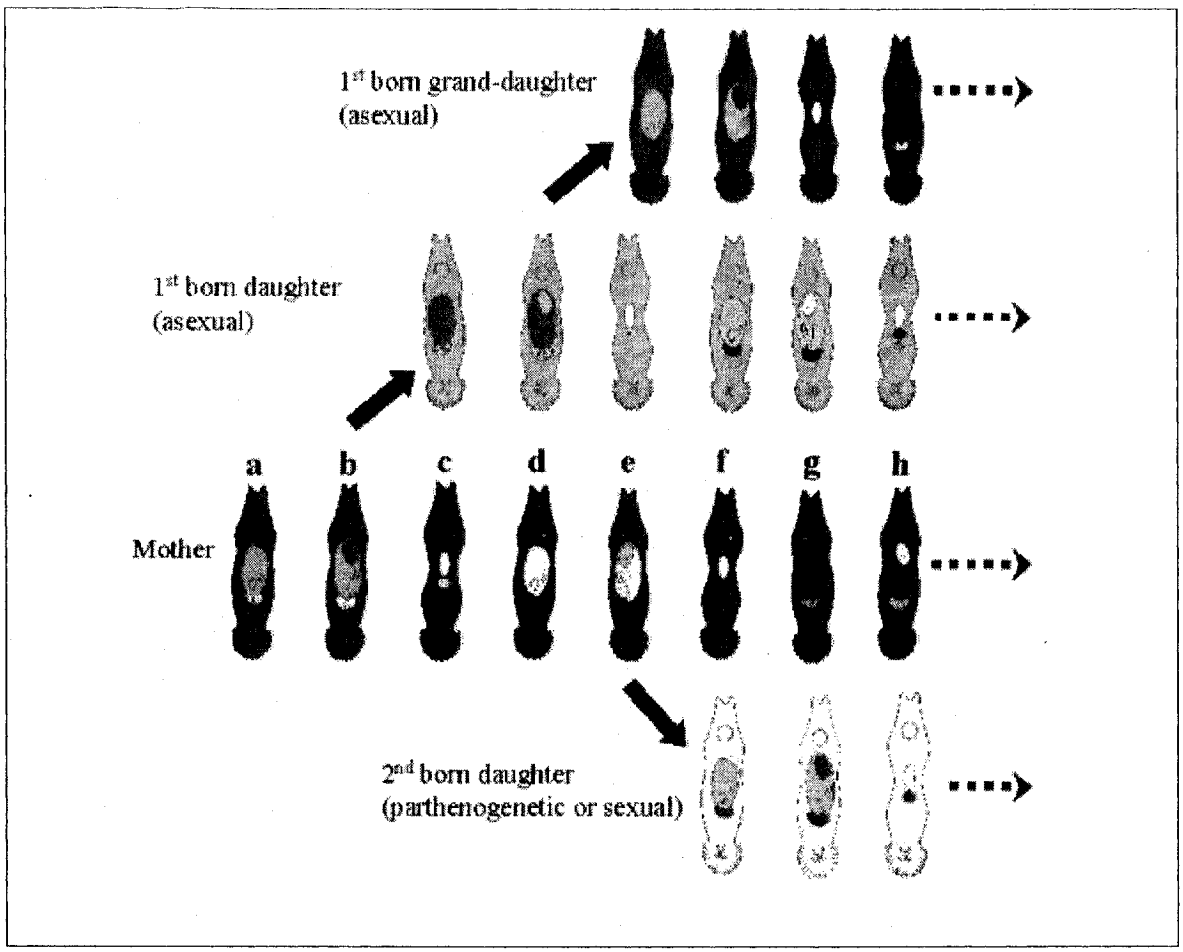


Fig. 2.3 *Gyrodactylus* life cycle. Different developmental stages of a newborn *Gyrodactylus* sp. (a-h) and its offspring. a-h represent the different stages of a newborn parasite. When this parasite is born it already contains a developing F1 embryo *in utero* and as the parasite matures an F2 embryo is visible developing within the F1. After birth of the 1st born daughter, an oocyte enters the uterus and development of the 2nd born daughter begins. The 1st born daughter can only develop asexually as development begins before birth of its mother, but 2nd and subsequent daughters may develop parthenogenetically or sexually. (courtesy of Dr. Joanne Cable, http://www.cf.ac.uk/biosi/research/biodiversity/staff/jc.html)

Moreover, Osland *et al.* (2006) found that *G. salaris* are not only able to survive on their dead hosts, but also they are able to feed and to maintain their virulence and pathogenicity, as salmon exposed to dead infected hosts attained higher parasite burdens than when exposed to living infected hosts. In contrast, *G. turnbulli* individuals leave the recently dead hosts, moving onto the water film, from where they can colonize new hosts (Cable *et al.*, 2002a). The number of parasites transferring onto the uninfected fish increases with parasite intensity. Furthermore, Scott and Anderson (1984) suggested that 30-40% of the parasites are able to survive host death by re-attaching to live fish.

*Gyrodactylus* are browsers, ingesting both dermal mucus and epidermal cells, while they move on the body surface of their host (Buchmann and Lindenstrøm, 2002). Digestion is accomplished in the digestive tract consisting of the mouth, pharynx, esophagus and gut. There is no anus. In addition, they may supplement their diet with organic nutrients of low molecular weight absorbed directly from water across the tegument, as other monogenean parasites do (Smyth and Halton, 1983). *Gyrodactylus* embryos *in utero* are probably fed by the transfer of nutrients from the gastrodermis through the uterine wall which is situated close to the gut (Cable *et al.*, 1996, 2002b; Jones *et al.*, 1998).

### *2.3.1 Gyrodactylus in guppies*

Two species of gyrodactylids parasitize the skin of guppies, and they are distinguishable on the basis of morphology (mainly the size and shape of the hard parts of the opisthaptor) and initial location on the host: (1) *Gyrodactylus bullatarudis* (Turnbull, 1956) - mainly on the anterior part of the body of the host,

and (2) *G. turnbulli* (Harris, 1986) – mainly on the caudal peduncle and caudal fin, with fewer on the pectoral, dorsal, pelvic and anal fins (Harris, 1988). However, the spatial distribution of parasites on the host changes during an infection, perhaps reflecting primarily random colonization of the fins followed by migration to the peduncle, and subsequent dispersal during the decline of the infection (Harris, 1988). A number of investigations have shown that *G. turnbulli* is the species usually found on guppies in the aquarium trade (i.e. Scott, 1982; Harris, 1986, 1988; Richards and Chubb, 1996, 1998).

In *G. turnbulli*, the average life span is 4.5 days for the attached parasites, whereas the detached individuals show vigorous longitudinal movements (Cable *et al.*, 2002a), but are not able to survive more than few hours, if they fail to locate a new host (Scott and Anderson, 1984). The instantaneous death rate increases with age, thus less than 2% of individuals survive to give birth three times (Scott, 1982).

### *2.3.2 Gyrodactylus and environmental factors*

As for all aquatic ectoparasites, *Gyrodactylus* is affected by abiotic factors in the surrounding environment, as reported by several authors. (Soleng *et al.*, 1999; Poléo *et al.*, 2004; Pettersen *et al.*, 2006). Physico-chemical conditions of water influence occurrence, life-span, reproduction, morphometrics and transmission of *Gyrodactylus*. The occurrence of *Gyrodactylus* seems to be affected by humus content, degree of eutrophication, temperature and salinity (Malberg, 1957, 1970 cited by Poléo *et al.*, 2004; Harris, 1980; Scott and Nokes, 1984; Jansen and Bakke, 1991; Appleby and Mo, 1997; Soleng and Bakke, 1997;

Soleng *et al.*, 1998). Across all these studies, the most important factor affecting *Gyrodactylus* spp. characteristics seems to be the environmental temperature. The impact of temperature on *Gyrodactylus* survival and reproduction was shown by several authors (Harris, 1980; Scott and Nokes,1984; Jansen and Bakke, 1991). Their results prove that each species has its own specific temperature preferences. For example, both survival and reproduction of *G. turnbulli* are water temperature-dependent (Scott and Nokes, 1984). They showed that although the longest average life span (5.5 days) was reached at 21°C, the highest fecundity (1.73 offspring) occurred at 25.5°C, and the highest instantaneous *per capita* birth rate at 27.5°C. Moreover, the parasite could not survive at 30°C, but was able to survive at the lowest temperature tested (17°C).

In addition, because of this preferred temperatures, there are seasonal differences in the parasite occurrence reflected in infection prevalence and intensity. For example, Dávidová *et al.* (2005) recorded in *Rhodeus sericeus* (a cold water fish) an increase in prevalence and intensity of infection with *G. rhodei*, as the temperature decreased. The highest values were recorded from January to April. One possible explanation could be that at higher temperatures the host response is more effective, as shown by Harris (1980). Temperature has been also reported to influence parasite morphometrics, particularly the size of the hard parts of the haptor (Geets *et al.*, 1999). Thus the specimens collected during the summer had significantly smaller values than specimens of the same species collected during the winter.

Another important category of environmental factors that was shown to affect gyrodactylids, is the one reflecting the antropogenic activity, such as heavy metals (aluminum, copper, zinc, iron, manganese), water pH and petroleum aromatic hydrocarbons (Khan and Kiceniuk, 1988; Soleng *et al.*, 1999, 2005; Poléo *et al.*, 2004; Pettersen *et al.*, 2006). Whereas aluminum, zinc and acidic water had a negative impact on gyrodactylids (Soleng *et al.*, 1999, 2005; Poléo *et al.*, 2004; Pettersen *et al.*, 2006), copper, iron, manganese and petroleum aromatic hydrocarbons seem to be beneficial for the parasites, as their numbers, or the prevalence of infection increased in exposed fish (Khan and Kiceniuk, 1988; Poléo *et al.*, 2004).

### *2.3.3 Population dynamics*

Due to the combination of viviparity, the embryonic development, and autoinfection (the daughters attach immediately after birth to the same host as their mother – Scott, 1982), *Gyrodactylus* numbers undergo rapid exponential growth even on an isolated fish. Depending on the parasite population dynamics following an initial infection, one of three outcomes is possible: the parasite population does not establish; parasite populations grow exponentially, then decrease to become extinct; parasite population continues to grow exponentially until it kills the fish. The time frame and the parasite peak burden are very variable even within the same group of fish.

This high variability of parasite population dynamics and the fate of infection on individual hosts could be a consequence of initial infectious dose, stochastic processes of parasite birth and death at low parasite numbers, host

genetics (Scott, 1982, 1985; Van Oosterhout *et al*, 2006), environmental factors (Scott and Nokes, 1984), parasite species, and host response (Richards and Chubb, 1998; Harris *et al.*, 2000). For instance, Richards and Chubb (1998) noted that *G. bullatarudis* were more susceptible than *G. turnbulli* to the host response. Whereas *G. bullatarudis* population increased and then declined to extinction after 40 days on adult guppies, *G. turnbulli* maintained low-intensity infection on 60% of adult fish for over 94 days and did not become extinct over the course of the experiment. They attributed these results to the weak immune response of the guppies to *G. turnbulli*, which allows the survival of small numbers of parasites, even without addition of naïve susceptible hosts. This finding contrasts with the results of Scott and Anderson (1984), who demonstrated that *G. turnbulli* are unable to persist within populations of young guppies in the absence of the constant influx of naïve susceptible fish. After the first epidemic, in the absence of fish immigration, the parasite population goes extinct either due to the low fish densities after the epidemic and/or the unsuitability of the available fish as hosts. With regular addition of susceptible fish, the parasite population causes recurrent epidemics with the average burden of parasites, the amplitudes of the cycles in parasite abundance and the intervals between peak abundances directly dependent on the rate of the input of susceptible hosts (Scott and Anderson, 1984). The authors attributed these oscillatory cycles to the occurrence of a temporary, partial refractory period to reinfection.

To understand the biological basis of the cyclic behavior, Scott (1985) investigated the infection dynamics on isolated guppies during the refractory

period following natural recovery of an initial infection. By reinfecting the recovered fish immediately or after 1, 2, 4, 6 weeks post-recovery, Scott (1985) found that peak parasite burdens were significantly lower, and occurred significantly earlier at all challenge periods, when compared the paired initial infections of controls. Challenge infection had a significantly shorter duration at 0, 1, 2 weeks post recovery compared with the paired control initial infection, suggesting that the refractory period following an initial infection is temporary, and that fish regain their full susceptibility within 4-6 weeks.

Another experiment was designed by Scott and Robinson (1984) to investigate the effect of the duration of a primary infection on challenge infection on guppies. One or 2 weeks after an initial infection, the infected fish were treated to kill the parasites, and immediately challenged. Monitoring the parasite population dynamics, the researchers noticed that *G. turnbulli* did not undergo the same growth and decay characteristics during a challenge infection as during the initial infection. Regardless of the duration of the primary infection, establishment of the challenge infection was lower, the peak parasite burden was lower and occurred earlier, and the duration of the infection was shorter, compared with the initial infection. Even a very short primary infection of 3 days provided some protection against challenge infection and caused the parasites to locate in previously unoccupied body regions of the host (Richards and Chubb, 1996). These differences between the population dynamics during initial and challenge infection may be due to an acquired, non-sterile immunity, and indicate that even an initial infection of only 3 days duration may be enough to confer protection to a challenge infection.

*2.3.4 Pathology*

The degree of pathology depends on the host and gyrodactylid species, the parasite intensity, the size of the fish, and environmental factors (i.e. temperature, crowding and poor sanitation). Infected guppies manifest altered swimming behavior. They are unable to maintain their vertical position and frequently weave from side to side. During their exploratory behavior, guppies are more attracted to come in close contact with heavily infected, or moribund fish, exhibiting a different behavior, or even dead ones (Cable *et al.*, 2002a). These behavioral changes may be important in the perception of infected individuals by the other fish, and increase transmission between hosts. Infected fish produce an increased amount of mucus that either detaches from their skin (Scott and Anderson, 1984), or tends to hinder movement of the fish because fish are not able to extend their fins. Richards and Chubb (1996) noticed in some guppies infected with *G. turnbulli* that the caudal peduncle became white and translucent because of an epidermal thickening that returned to normal after recovery from infection. The coloration of infected males is reduced (Kennedy *et al.*, 1987) and females avoided them (Lopez, 1999). Similarly, Heggbert and Johnsen (1982) reported skin depigmentation, an increased mucification, and secondary infections in parr of *S. salar* in Norwegian rivers infected with *G. salaris*. Cone and Wiles (1989) reported microscopic patches of necrotic tissue induced by the attachment of *G. colemanensis* to superficial epithelial cells of rainbow trout, but due to the continuous movement of the parasites, the necrosis did not develop into localized lesions. Morbidity and mortality can be caused directly by the parasite, or through secondary infections with protozoa, bacteria or fungal

parasites (Cone, 1999). Heavily infected fish become moribund and die. Some individuals can get rid of their parasites and recover (Scott, 1985).

### 2.3.5 Treatment

The treatment of choice for guppies consists of a bath with a weak solution of formalin (1:4000) for 1h (Scott, 1984). Very sick fish do not tolerate formalin well and all fish should be carefully watched during chemical administration. If adverse reaction is observed, fish should be removed from the treatment tank at once and placed in clear water (Reed *et al.*, 1998). Other drugs also effective against *Gyrodactylus*, but more toxic for the fish, are potassium permanganate, praziquantel, niclosamide and levamisole-HCL (Cone, 1999). Recently, Steverding *et al.* (2005), showed the potential use of 3 and 30 ppmv (parts per million by volume) TTO (Australian tea tree - *Melaleuca alternifolia*) oil in combination with 0.01 % Tween 80 as an effective treatment of *Gyrodactylus* spp. infection.

### *2.3.6 Host response against gyrodactylids (Monogenea)*

Various studies on gyrodactylid infections have demonstrated that fish are able to mount temporary acquired protection against these ectoparasites (Lester and Adams, 1974; Scott, 1985; Richards and Chubb, 1996, 1998; Buchmann and Bresciani, 1998, 1999). Moreover, several authors indicated the presence of cross-protection when the same fish was successively infected with different species of gyrodactylids, or *Gyrodactylus* and other parasites. Following initial infections with one or the other of two different species of *Gyrodactylus* in

guppies, Richards and Chubb (1996) reinfected guppies with *G. turnbulli* or *G. bullatarudis*. Despite the fact that the authors used only 3-day primary infections, they concluded that the response of guppies to an initial *Gyrodactylus* infection provides some protection against a challenge infection, regardless of whether the same or a different species of parasite was used in the initial and challenge infections. Buchmann *et al.* (1999) provided other evidence indicating the cross-protection of the fish previously infected with *Gyrodactylus*. When exposed to *Ichthyophthirius multifillis* infections, cured fish previously infected with *G. derjavini* became less heavily infected with the protozoan and their mortality rate was lower than that of naïve fish. Nevertheless, the exact nature of the host response to *Gyrodactylus* infection remains unknown. However, due to the fact that gyrodactylid infections have been associated with hypersecretion of mucus and /or a localized epithelial cell hyperplasia by fish, the host response seems to be predominantly local. Several studies have indicated the crucial importance of epidermal mucous cells in the host response against gyrodactylid infections, and both the quantity of these cells (e.g. Wells and Cone, 1990; Linderstrøm and Buchmann, 2000), as well as various humoral and cellular factors (e.g. Buchmann and Bresciani, 1998, 1999; Buchmann, 1999; Harris *et al.*, 1998, 2000) seem to be involved in these skin reactions.

Lester and Adams (1974) first reported the 'cuticular shedding' during stickleback infection with *Gyrodactylus alexandri*, as a response to eliminate the parasite. Similar responses have been reported in infected guppies (Scott and Anderson, 1984; Richards and Chubb, 1996). Across various studies, results suggest thickening of the epithelium through increase in the number of cell layers

(Appleby *et al.,* 1997) and hyperplasia of epithelial cells (Wells and Cone, 1990), and change in mucous cell density through the course of infection. In rainbow trout infected with *G. derjavini,* after an initial hyperplasia, a significant depletion of approximately 50% of the mucous cell density occurred (Lindenstrøm and Buchmann, 2000). Similarly Wells and Cone (1990) reported decreased mucous cell density in rainbow trout experimentally infected with *G. colemanensis* and *G. salmonis.* In contrast, Lindenstrøm and Buchmann (2000) suggested that gyrodactylids induce goblet cell proliferation eventually leading to hyperplasia. The excessive mucus and the high rate of epithelial proliferation are unfavorable for the parasites as they are removed *via* sloughing of mucus and/or cells. With time, the epidermis damage caused by the flukes together with the continuing discharge of mucus from mucous cells to combat the infection seems to exhaust the goblet cells, which are consequently depleted (Wells and Cone, 1990). Moreover, Buchmann and Bresciani (1998) noticed that *G. derjavini* seems to avoid the areas rich in mucous cells in the later phases of the infection.

In their *in vitro* studies, Buchmann *et al.* (2000) showed that epithelial cells are able to interact, encapsulate and degrade *G. derjavini.* Furthermore, Sigh and Buchmann (2000) studied the importance of epidermal putative mast-cells (metachromatic thionin-positive cells) in trout infected with *G. derjavini.* They found a marked degranulation and decrease in the density of these cells during infection, which suggest their involvement in antiparasitic defense.

While gyrodactylids browse on infected fish epidermis, they ingest both mucus and epidermal cells. The opisthaptoral hooklets induce mechanical disruption of the epithelium, exposing the parasite to both mucus and epidermal

cell constituents. Buchmann and Bresciani (1998) showed that fish epithelium and mucus contain complement and 'therefore gyrodactylids live and feed in a complement-rich environment'. They found that the only consistent requirement for parasite killing *in vitro* is complement, *via* alternate pathway. When complement was inactivated by heat or EDTA, parasite killing was lost (Harris *et al.*, 1998). In fact, using an immunocytochemical assay, Buchmann (1998) reported that complement factor 3 bound directly to the openings and glands of the anterior extremity, to the hamulus sheath and to the body surface of *G. derjavini*. The author suggested that carbohydrate epitopes on the parasite activated C3 complement. Buchmann and Bresciani (1999) noticed that macrophages from rainbow trout attached *in vitro*, especially to the openings of the anterior extremity of living *G. derjavini*. They speculated that this effect might be induced by complement factor C3 released by activated macrophages. They concluded that during host response, macrophages in fish skin may be responsible for increased complement levels in trout skin, and for IL-1 release, which, in turn, induces host epidermis hyperplasia and enhanced mucus secretion. However, there is no evidence so far that macrophages bind to the parasite *in vivo*.

However, indirect proof that fish mount a non-specific immune response against gyrodactylid infections is offered by experiments done by Harris *et al.* (2000). Using immunosupressing implants with hydrocortisone acetate (an immunosuppressant with similar action to cortisol, which may downregulate complement activity and suppress inflammatory responses), they increased the susceptibility of a variety of salmonid species to *G. salaris*. Similarly, Olafsdottir

and Buchmann (2004) showed that treatment with dexamethasone (a corticosteroid restraining IL-1β expression in trout cells) increased the susceptibility of Atlantic salmon to infection with *G. derjavini*, through a significant increase in mucous cell densities, which, in turn, could account for either a decreased anti-parasitic mucus efficacy, or a parasite preference for the intact mucous cells.

To date, no parasite-specific immunoglobulins have been reported to be involved in the host response against *Gyrodactylus* infection. Buchmann (1998, 1999) found no binding of immunoglobulins from infected rainbow trout to the *G. derjavini* surface, but the author did not exclude the possibility that antibodies bind to molecules in the parasite internal structure. Furthermore, passive immunization with native immune sera (with active complement), or heat-inactivated immune sera (with heat-inactivated complement), or non-immune sera (with heat-inactivated complement), did not confer even partial protection against infection with *G. derjavini* in naïve fish (Lindenstrøm and Buchmann, 2000). The authors concluded that the elimination of *G. derjavini* is not primarily attributable to a systemic humoral component, although they speculated that the transferred components might be rapidly degraded in the recipients. In addition, the great mobility of gyrodactylids and their movement onto different fish and the substrate may reduce the impact of complement. In fact, although *G. salaris* is affected by activated complement in salmon serum and mucus *in vitro*, the parasite seems to be unaffected, and also it multiplies and often kills the host *in vivo*.

## 2.4 Zinc in fish

### *2.4.1 Source*

Zn is one of the most common elements in the earth's crust. Most Zn found naturally in the environment is in the form of zinc sulfide. Zn enters the air, water, and soil as a result of both natural processes (erosion of the mineral deposits) and human activities (mining, metallurgical, cosmetics and pharmaceutical industries). Also, as Zn is a required trace element, it is added as a growth factor in farm animal food (Creech *et al.*, 2004). Waste waters from manufacturing of Zn and other metal and Zn chemical industries, domestic and farm waste water and run-off from soil containing Zn can discharge Zn into waterways (*Toxicological Profile for Zinc May 1994 Update* - Agency for Toxic Substances and Disease Registry United States Public Health Service). Moreover, in fish farms unconsumed growth-enhancing nutritional supplements containing Zn will increase even further the concentration of waterborne Zn due to poor uptake of this element (Wekell *et al.*, 1983)

In aquatic systems, most Zn is found in the sediments whereas in the water column, Zn is almost entirely particulate and coupled with dissolved organic and inorganic compounds (Florence *et al.,* 1992; Rozan *et al.*, 2000). The level of dissolved Zn in water may increase as the acidity of water increases. Canadian Water Quality Guidelines (CWQG) for aquatic biota, including fish, indicate less than 30 µg/L as an acceptable Zn concentration in water.

It has been reported that Zn occurs at low concentrations in freshwaters (20µg/L) due to natural watering of mineral deposits (Handy, 1996), but in industrialized areas waterborne Zn concentration can exceed 100 µg/L. Seawater

collected from the open ocean may contain lower levels of total Zn (10-600 ng/L), but in polluted coastal waters with discharge of industrial effluents Zn can reach concentrations of 20-30000 µg/L (Handy, 1996).

### *2.4.2 The physiology and toxicology of Zn in fish*

For aquatic organisms, Zn is both an essential micronutrient (Watanabe *et al.,* 1997) and, at high concentrations, an important toxicant in polluted environments (Widianarko *et al.*, 2000, 2001).

#### 2.4.2.1 Zn as an essential micronutrient.

Life without Zn is unlikely. As a vital micronutrient, Zn is present in every cell of the body, and is involved in the structure or function of more than 300 enzymes and proteins, some governing the function of DNA, others participating in the metabolism of nucleic acids, proteins, carbohydrates and fatty acids (Vallee and Falchuk, 1993). Zn is involved in three kinds of functions: catalytic, structural and regulatory (Cousins, 1998). Many cellular processes such as protein synthesis, cell growth, apoptosis, antioxidant enzyme superoxidase dismutase activity, are Zn-dependent. Zn also plays important roles in growth and development, in the immune response in terms of increase resistance to infection and tumor growth, antiviral proprieties, in neurological function and in reproduction. So, depending on species of fish, Zn dietary requirements are 15-40 mg/kg. If these requirements are not met, Zn deficiency occurs, leading to physiological perturbation of growth, reproduction, vision and immunity (Watanabe *et al.,* 1997).

Fish are able to absorb Zn by two major pathways: the gill and the intestine. Waterborne Zn absorption through the gills is influenced by water physico-chemical characteristics. Thus, Zn-absorption rate and accumulation into the gill tissues is decreased by increased water hardness, elevated cadmium content, or by low water pH (Hogstrand and Wood, 1996). The brachial pathway for waterborne Zn takes place through an apical $Ca^{2+}$ channel in the chloride cells of gills and may influence both Zn uptake and potential lethal hypocalcaemia induced by Zn exposure (Hogstrand *et al.,* 1996, 1998). Dietary Zn uptake is likely to be the major source for Zn assimilation, because of the intestine's low affinity for $Zn^{2+}$, and its high capacity for $Zn^{2+}$ transport (Woodward *et al.*, 1994; Glover and Hogstrand, 2002). Digestive secretions may extract Zn from food and ingested particulate matter (Powell *et al.,* 1999), increasing its bioavailability. According to Wekell *et al.* (1983), dietary Zn supplementation increased the total amount of body Zn in a dose-dependent manner in freshwater rainbow trout (*Oncorhynchus mykiss*). Shears and Fletcher (1983) hypothesized that Zn passage across the basolateral membrane is passive.

From the gills and the intestine, blood transports Zn ions to various tissues. In the tissues, metals are distributed according to metabolic requirements and physiological mechanisms to manage any excess. Zn is concentrated into the storage tissues (Grober-van Heerden *et al.,* 1991), represented mainly by skin and bone (Nakano *et al.*, 1992), but may also accumulate in heart, brain, gills, kidney, liver and gut (Andres *et al.*, 2000). The mechanisms for maintaining Zn homeostasis are so well regulated that Zn deficiency occurs extremely rarely

in fish. Zn is normally excreted *via* kidney or chloride cells of the gills (Hogstrand and Wood, 1996).

2.4.2.2. Zn as an important toxicant.

Orally assimilated Zn may cause chronic toxicity (Woodward *et al.*, 1994), whereas brachial uptake is thought to be responsible for acute pathology (Hogstrand *et al.*, 1996, 1998). However, only excessive environmental Zn is considered to be toxic, seriously affecting aquatic organisms, with lethal or sublethal effects (Widianarko *et al.*, 2000, 2001).

Fish Zn sensitivity is highly variable. For instance, it depends on species of fish exposed, with Perciform fish being the most resistant, whereas the Clupeiforme group is the most sensitive. Water salinity influences Zn sensitivity as well, so that in general, freshwater fish are more sensitive to zinc than marine species (Eisler, 1993). Also developmental stage affects the sensitivity to Zn, embryos and larvae being the most sensitive. Gender influences Zn sensitivity as well, female guppies being reported to be more resistant than males to acute zinc toxicity (Eisler, 1993).

Acute lethality of dissolved Zn increases with decreasing water hardness and pH. Although typically 96 h $LC_{50}$ for waterborne Zn concentrations are 1-10 mg/L in soft water and 3-20 mg/L in hardwater, there are reported values ranging from 90 µg/L to 40 mg/L (Hogstrand and Wood, 1996).

Zn-induced behavioral changes (ventilation and cough rate; avoidance or attractance, activity, loss of appetite) occur at concentrations as low as 5.6 µg Zn/L (Atchinson *et al.*, 1987). Zinc poisoning in fish induced hyperactivity followed

by sluggishness before death, lethargic and uncoordinated surface swimming, hemorrhage on gills and at the base of fins, shed scales, and extensive body and gill mucous (Eisler, 1993).

Impairment of reproduction is the most sensitive effect of Zn exposure. In soft water, concentrations up to 50 µg Zn/L impair fish reproduction through the reduction of the number of spawnings and eggs produced per female (Hogstrand and Wood, 1996).

Elevated concentrations of waterborne Zn induce disruption of gill tissue, disturbance of acid-base and ionic regulation (e.g., impairment of brachial uptake of $Ca^{2+}$), and hypoxia (Köck and Bucher, 1997), proliferation or enhanced turnover of ionocytes (Hogstrand and Haux, 1991; Roesijadi, 1992), displacement of other metals from various enzymes (Roche and Boge, 1993), interactions with ion-transport channels (ATP-ases) in all membranes (McGeer *et al.,* 2000), and increased production of metallothioneins (Borghesi and Lynes, 1996). Sanchez-Dardon *et al.* (1999) showed immunosuppression of phagocytic activity of macrophages in rainbow trout exposed for 30 days to 10 and 50 µg Zn/L, but not to 30 µg Zn/L. In contrast, in zebrafish exposed to 50 µg/L of Zn for 7 days, Rougier *et al.* (1994) found significantly increased phagocytic activity, suppression of non-specific cytotoxic cell activity, and a significant decrease in the proportion of leukocytes. However, following exposure of fish to Zn polluted waters, Iger *et al.* (1994) also noticed leukocyte invasion of the dermis and epidermis. These conflicting results could be attributed either to the different sensitiveity of the species tested, concentration tested, duration and/or route of exposure.

2.4.2.3. Detoxification and acclimation to elevated Zn exposure.

The survival of aquatic organisms in Zn polluted waters depends on the homeostatic control of both mechanisms of the absorption of Zn and mechanisms for limiting its assimilation and/or toxicity. After absorption, the organism must either eliminate it through excretion or sequester it to prevent toxicity (Sauer and Watabe, 1984). Sauer and Watabe (1984, 1989) showed that fish scales incorporated Zn into the mineralized osseous layer. After 8 weeks of exposure at 10 µg Zn/L, 'lysosomal structures' of osteoblasts had a dramatic increase in the metal content, suggesting that these organelles are involved in the accumulation of heavy metals by fish scales, as part of storing and/or detoxification process. Furthermore, waterborne Zn exposure induced production of metallothionein (MT), a small intracellular metal-binding protein that contains cysteine-rich polypeptides involved in metal chelation, that plays an important role in detoxification of both essential (Zn and Cu), and nonessential (Cd, Hg, Ag) heavy metals (Hogstrand and Haux, 1991). The percent of Zn bound to MTs in fish liver increases substantially when fish are exposed to Zn (Hogstrand and Haux, 1990; Hogstrand *et al.*, 1991). Moreover, MT synthesis was shown to be directly induced by metals (Cu, Zn, Cd, Hg, Ag).

Fish surviving chronic, sublethal exposure to Zn (and other metals) is able to correct the ionic disturbance, and to acquire physiological acclimation. After a short period of physical damage and disruption of physiological homeostasis, a recovery period follows, when tissue repair begins and synthesis of MTs is upregulated, thus re-establishing a homeostatic equilibrium with increased tolerance to the metal (McGeer *et al.*, 2000). For instance, a 2.5 times increased

$LC_{50}$ was recorded in 5 day-Zn-exposed trout, compared with unexposed fish (Bradley *et al.*, 1985).

2.4.2.4 Guppies response to elevated Zn exposure

Widianarko *et al.* (2000, 2001) found that guppies did not avoid highly polluted sites, and are well established in polluted water. Moreover, they noticed significant differences in body concentrations of Zn between fish collected from sites with different degrees of pollution. The body metal concentrations were not influenced by the fish size, but were correlated with the concentrations in sediments. These suggest the guppies tolerate Zn, rather than regulate its uptake/excretion. The authors concluded that guppies from urban streams are a good bioindicator for urban metal pollution of sediments and at the same time, they are useful test animals in aquatic toxicity experiments.

*2.4.3 Zn toxicology in aquatic parasites*

The toxic effects of pollutants (e.g. heavy metals) on aquatic parasites have been a subject of increasing interest in recent years. Several studies concluded that fish parasites generally reflect environmental disturbances (Khan and Thulin, 1991; Mackenzie *et al.*, 1995), and may be useful as bioindicators (Sures, 2002).

To date, almost all the toxicological studies in trematodes were done on larval digeneans (miracidia, cercariae, metacercarie) in terms of their survival, activity, free encystment, selective binding of pollutants to various parasitic structures, and host location behavior. Depending on the developmental stage

and the concentration of toxicant, the effects were either detrimental or, on the contrary, beneficial because of the impairment of host response (Table. 1).

To date there are only few reported studies on the impact of various metals (Al, Cu, Zn, Fe, Mn) on *Gyroactylus*. Thus, it has been demonstrated that Al and Zn exposure in concentrations ranging from 50 to 200 µg Al/L and 50 to 400 µg Zn/L respectively, had a negative impact only in *G. salaris* infections and did not affect Atlantic salmon, its host (Soleng *et al.*, 1999; Poléo *et al.*, 2004).

Table 2.1: Waterborne Zn effects on larval digeneans

| Parasite | Stage | Toxicant | Dose | Effect | Suggested Mechanism | References |
|---|---|---|---|---|---|---|
| *Schistosoma mansoni* | Miracidia at maximum infectivity upon hatching | Cd | 10 µg/L | Survival ↑ Activity ↓ | Inhibition of utilization of glycogen reserves / Enzymatic inhibition of glycogen synthesis | Morley *et al.*, 2001a |
| | | Zn | | | | |
| | | Cd | 100 µg/L | Survival ↓ | | |
| | | Zn | | | | |
| | | Cd and Zn | 10 µg/L | Survival ↓ | Cd and Zn are antagonistic | |
| | Miracidia after maximum infectivity upon hatching | Cd | 10000 µg/L | Survival ↓ | Metal toxicity ↑ | |
| | | Zn | | | | |
| | Miracidia | Cd | 1000 µg/L | Avoidance behavior Sensorial host location and infection ↓ Transmission ↓ | Metal bound to skin sensory structures | |
| | | Zn | | | | |
| | | Cd and Zn | 100 µg/L | | | |
| | | Zn | 10000 µg/L | Metal accumulation ↑ | | |
| | Cercariae | Zn | 6810 µg/L | Survival ↓ | Disrupted tegumental integrity | Ash and Dresden, 1977 |
| | | | | Penetration ↓ Sensorial host location and infection ↓ Transmission ↓ | Proteases inhibition | |

Table 2.1 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| *Echinoparyphim recurvatum* | Cercariae | Zn | 100 µg/L | Survival ↓<br>Infectivity ↓<br>Swimming ↓<br>Transmission ↓ | Enzymatic inhibition<br>Enzymatic inhibition of penetration gland secretions | Evans, 1982 |
| | | Cd | 10 µg/L | Orientation behaviour ↓<br>Transmission ↓ | Metal bound to skin sensory structures | Morley *et al.*, 2003b |
| | | Zn | | | | |
| | | Cd | 1000 µg/L | Sensorial host location and infection ↓<br>Transmission ↓ | | |
| | | Zn | | | | |
| *Diplostomum spathaceum* | Cercariae | Cd | 0.1-100 µg/L | Survival ↓ with concentration ↑ | Depletion of glycogen reserves | Morley *et al.*, 2001b |
| | | Zn | | | | |
| | | Cd and Zn | 0.1-100 µg/L | Survival ↑ | Cd and Zn are antagonistic<br>Inhibition of utilization of glycogen reserves | Morley *et al.*, 2002 |
| | | Zn | 0.1 µg/L for 3 h | Activity ↓<br>Transmission ↓ | Inhibition of utilization of glycogen reserves | Morley *et al.*, 2003a |
| | | | 0.1 µg/L for 24 h | | | |
| *Cryptocotyle lingua* | Cercariae | Zn | 1000-2000 µg/L | Swimming / Transmission ↓<br>Survival ↓ | Metal accumulation ↑ | Cross *et al.*, 2001 |

## References


Agency for Toxic Substances and Disease Registry United States Public Health Service - Toxicological Profile for Zinc, May 1994 Update.

Andres, S., Ribeyre, F., Tourencq, J.N. and Boudou, A., 2000. Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). *Sci. Tot. Environ.* 248: 11-25.

Appleby, C., Mo, T.A. and Aase, I.L., 1997. The effect of *Gyrodactylus salaris* (Monogenea) on the epidermis of Atlantic salmon, *Salmo salar* parr in the river Batnfjordselva, Norwy. *J. Parasitol.* 83: 1173-1174.

Asch, H.L. and Dresden, M.H., 1977. *Schistosoma mansoni*: effects of zinc on cercarial and schistosomule viability. *J. Parasitol.* 63: 80-86.

Atchison, G.J., Henry, M.G., and Sandheinrich, M.B., 1987. Effects of metals on fish behavior: a review. *Environ. Biol. Fish.* 18: 11-25.

Bakke, T.A., Harris, P.D. and Cable, J., 2000. Host specificity dynamics: observations on gyrodactylid monogeneans. *Int. J. Parasitol.* 32: 281-308.

Bakke, T.A., Harris, P.D., Hansen, L.P. and Jansen, P.A., 1992. Host specificity and dispersal strategy in gyrodactylid monogenean with particular reference to *Gyrodactylus salaris* (Plathyhelminthes, Monogenea). *Dis. Aquat. Org.* 13: 45-57.

Bakke, T.A., Jansen, P.A. and Hansen, L.P., 1990. Differences in the host resistance of Atlantic salmon, *Salmo salar* L., stocks to the monogenean *Gyrodactylus salaris* Malmberg, 1957. *J. Fish Biol.* 37: 577-587.

Bakke, T.A., Jansen, P.A. and Harris, P.D., 1996. Differences in susceptibility of anadromous and resident stocks of Artic charr to infections of *Gyrodactylus salaris* under experimental conditions. *J. Fish Biol.* 49: 341-351.

Borghesi, L.A. and Lynes, M.A., 1996. Nonprotective effects of extracellular methallothionein. *Toxicol. Appl. Pharmacol.* 139: 6-14.

Bradley, R. W., DuQuesnay, C. and Sprague, J. B., 1985. Acclimation of rainbow trout, *Salmo gairdneri* Richardson, to zinc: kinetics and mechanism of enhanced tolerance induction. *J. Fish Biol.* 27(4): 367-379.

Buchmann, K., 1998. Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). *Dis. Aquat. Org.* 32: 195-200.

Buchmann, K.; 1999. Immune mechanisms in fish skin against monogeneans – a model. *Folia Parasitol.* 46: 1-9.

Buchmann, K. and Bresciani, J., 1998. Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*: association between mucous cell density in skin and site selection. *Parasitol. Res.* 84: 17-24.

Buchmann, K. and Bresciani, J., 1999. Rainbow trout leukocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini. Dis. Aquat. Org.* 35: 13-22.

Buchmann, K. and Lindenstrom, T., 2002. Interactions between monogenean parasites and their fish hosts. *Int. J. Parasitol.* 32: 309-319.

Buchmann, K., Lindenstrom, T. and Sigh, J., 1999. Partial cross protection against *Ichthyophthirius multifillis* in *Gyrodactylus derjavini* immunized rainbow trout. *J. Helminthol.* 73: 189-195.

Buchmann, K., Nielsen, C.V. and Bresciani, J., 2000. *In vitro* interactions between epithelial cells and *Gyrodactylus derjavini. J. Helminthol.* 74: 203-208.

Cable, J., Harris, P.D., 2002. Gyrodactylid developmental biology: historical review, current status and future trends. *Int. J. Parasitol.* 32: 255-280.

Cable, J., Harris, P.D. and Tinsley, R.C., 1996. Ultrastructural adaptations for viviparity in the female reproductive system of gyrodactylid monogeneans. *Tiss. Cell.* 28: 515-526.

Cable, J., Harris, P.D. and Tinsley, R.C., 1998. Life history specializations of monogenean flatworms: a review of experimental and microscopical studies. *Microsc. Res.Tech.* 42: 186-199.

Cable, J., Scott, E.C.G., Tinsley, R.C. and Harris, P.D., 2002a. Behavior favoring transmission in the viviparous monogenean *Gyrodactylus turnbulli. J. Parasitol.* 88: 183-184.

Cable, J., Tinsley, R.C. and Harris, P.D., 2002b. Survival, feeding and embryo development of *Gyrodactylus gasterostei* (Monogenea: Gyrodactylidae). *Parasitology* 124: 53-68.

Canadian Council of Ministers of the Environment, 2005. Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life. http://www.ccme.ca/assets/pdf/wqg_aql_summary_table.pdf.

Cone, D.K., 1999. Monogenea. In *Fish Diseases and Disorders. Vol. 1. Protozoan and Metazoan Infections* (ed. Woo, P.T.K.), pp. 289-327. CABI Publishing, Wallingford, UK.

Cone, D.K. and Wiles, M., 1989. Ultrastructural study of attachment of *Gyrodactylus colemanensis* (Monogenea) to fins of fry of *Salmo gairdneri. Proc. Helminthol. Soc. Washington.* 56: 29-32.

Cousins, R.J., 1998. A role of zinc in the regulation of gene expression. *Proc. Nutr. Soc.* 57: 307-311.

Creech, B. L., Spears, J. W. , Flowers, W. L., Hill, G. M., Lloyd, K. E., Armstrong, T. A. , and Engle, T. E., 2004. Effect of dietary trace mineral concentration and source (inorganic vs. chelated) on performance, mineral status, and fecal mineral excretion in pigs from weaning through finishing. *J. Anim. Sci.*. 82: 2140–2147.

Cross, M.A., Irwin S.W.B. and Fitzpatrick, S.M., 2001. Effects of heavy metal pollution on swimming and longevity in cercariae of *Cryptocotyle lingua* (Digenea: Heterophidae). *Parasitology* 123: 499-507.

Cunningham, C.O. and Mo, T.A., 1997. Random amplified polymorphic DNA (RAPD) analysis of three Norwegian *Gyrodactylus salaris* populations (Monogenea; Gyrodactylidae). *J. Parasitol.* 83: 311-314.

Dávidová M., Jarkovský J., Matějusová I. and Gelnar M., 2005. Seasonal occurrence and metrical variability of *Gyrodactylus rhodei* Žitňan, 1964 (Monogenea, Gyrodactylidae). *Parasitol. Res.* 95: 398-405.

Dussault, G.V. and Kramer, D.L., 1981. Food and feeding behavior of the guppy, *Poecilia reticulata* (Pisces: Poeciliidae). *Can. J. Zool.* 59: 684-701.

Eisler, R., 1993. Zinc hazards to fish, wildlife and invertebrates: a synoptic review. U.S. Department of the Interior Fish and Wildlife Service. Patuxent Wildlife Research Center Biological Report 10. Contaminant Hazard Reviews Report 26. Laurel, Maryland 20708.

Emmens, C.W., 1970. *Guppy handbook* (ed.T.F.H. Publications), pp. 5-9, 23-41.

Evans, N.A., 1982. Effect of copper and zinc upon the survival and infectivity of *Echinoparyphium recurvatum* cercariae. *Parasitology* 85: 295-303.

Fletcher, T.C., Jones, R. and Reid, L., 1976. Identification of glycoproteins in goblet cells of epidermis and gill of plaice (*Pleuronectes platessa* L.), flounder (*Platichthys fleus* L.) and rainbow trout (*Salmo gairdneri* Richardson). *Histochem. J.* 8: 597-608.

Florence, T.M., Morrison, G.M. and Stauber, J.L., 1992. Determination of trace element speciation and the role of speciation in aquatic toxicity. *Sci. Tot. Environ.* 125: 1-13.

Geets, A., Appleby, C., and Ollevier, F., 1999. Host-dependent and seasonal variation in opisthaptoral hard parts of *Gyrodactylus* cf. *arcuatus* from three *Pomatoschistus* spp. and *G. arcuatus* from *Gasterosteus aculeatus*: a multivariate approach. *Parasitology* 119: 27-40.

Glover, C.N. and Hogstrand, C., 2002. *In vivo* characterization of intestinal zinc uptake in freshwater rainbow trout. *J. Experim. Biol.* 205: 141-150.

Grober-van Heerden, E., van Vuren, J.H.J. and du Preez, H.H., 1991. Bioconcentration of atrazine, zinc and iron in the blood of *Tilapia sparrmani* (Cichlidae). *Comp. Biochem. Physiol.* 100C: 629-633.

Handy, R.D., 1996. Dietary exposure to toxic metals in fish. In *Toxicology of Aquatic Pollution. Physiological, cellular and molecular approaches*. (ed. Taylor E. W., Department of Biological Sciences, University of Birmingham), pp. 29-60. Cambridge University Press, Cambridge.

Handy, R.D., Eddy, F.B. and Romain, G., 1989. *In vitro* evidence for the ionoregulatory role of rainbow trout mucus in acid, acid/aluminum and zinc toxicity. *J. Fish Biol.* 35: 737-747.

Harris, P., 1980. The effect of temperature upon population growth in the viviparous monogeneans *Gyrodactylus*. *Parasitology* 81: R26–R26.

Harris, P.D., 1985. Observations on the development of the male reproductive system in *Gyrodactylus gasterostei* Glaser, 1974 (Monogenea, Gyrodactylidae). *Parasitology* 91: 519-529.

Harris, P.D., 1986. Species of *Gyrodactylus* von Normann 1832 (Monogenea, Gyrodactylidae) from poeciliid fishes with a description of *G. turnbulii* sp.n. from the guppy *Poecilia reticulata* (Peters). *J. Nat. History.* 20: 183-191.

Harris, P.D., 1988. Changes in the site specificity of *Gyrodactylus turnbulli* Harris, 1986 (Monogenea) during infections of individual guppies *Poecilia reticulata* (Peters, 1859). *Can. J. Zool.* 66: 2854-2857.

Harris, P.D., 1989. Interactions between population growth and sexual reproduction in the viviparous monogenean *Gyrodactylus turnbulii* Harris, 1986 from the guppy *Poecilia reticulata* (Peters). *Parasitology* 98: 254-261.

Harris, P.D., Jansen, P.A. and Bakke, T.A., 1994. The population age structure and reproductive biology of *Gyrodactylus salaris* Malmberg (Monogenea). *Parasitology* 108: 167-173.

Harris, P.D., Soleng, A. and Bakke, T.A., 1998. Killing of *Gyrodactylus salaris* (*Platyhelminthes, Monogenean*) mediated by host complement. *Parasitology* 117: 137-143.

Harris, P.D., Soleng, A. and Bakke, T.A., 2000. Increased susceptibility of salmonids to the monogenean *Gyrodactylus salaris* following administration of hydrocortisone acetate. *Parasitology* 120: 57-64.

Heggberget, T.G. and Johnsen, B.O., 1982. Infections by *Gyrodactylus* sp. of Atlantic salmon, *Salmo salar* L., in Norwegian rivers. *J. Fish Biol.* 21: 15-26.

Hogstrand, C. and Haux, C., 1990. Metallothionein as an indicator of heavy-metal exposure in two subtropical fish species. *J. Experim.Marine Biol. Ecol.* 138: 69-84.

Hogstrand, C. and Haux, C., 1991. Binding and detoxification of heavy metals in lower vertebrates with reference to metalothionein. *Comp. Biochem. Physiol.* 100C: 137-141.

Hogstrand C., Lithner, G. and Haux, C., 1991. The importance of metallothionein for the accumulation of cooper, zinc and cadmium in environmentally exposed perch, *Perca fluviatilis. Pharmacol. Toxicol.* 68: 492-501.

Hogstrand, C. and Wood, C.M., 1996. The physiology and toxicology of zinc in fish. In *Toxicology of Aquatic Pollution physiological, cellular and molecular approaches*. (ed. Taylor E. W., Department of Biological Sciences, University of Birmingham), pp. 61-84. Cambridge University Press.

Hogstrand, C., Verbost, P.M., Wendelaar Bonga, S.E. and Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. *Am. J. Physiol.* 270: R1141-R1147.

Hongstrand, C., Webb, N. and Wood, C.M., 1998. Covariation in regulation of affinity for brachial zinc and calcium uptake in freshwater rainbow trout. *J. Exp. Biol.* 201: 1809-1815.

Iger, Y., Jenner, H. and Wendelaar Bonga, S.E., 1994. Cellular responses in the skin of rainbow trout (*Oncorhynchus mykiss*) exposed to Rhine water. *J. Fish Biol.* 45: 1119-1132.

Jansen, P.A. and Bakke, T.A., 1991. Temperature-dependent reproduction and survival of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes: Monogenea) on Atlantic salmon (*Salmo salar* L.). *Parasitology* 102: 105–112.

Johnsen, B.O. and Jensen, A.J., 1991. The *Gyrodactylus* story in Norway. *Aquaculture* 98: 289-302.

Jones, M.K.J., Ernst, I. and Whittington, I.D., 1998. The uterine epithelium of *Gyrodactylus kobayashii* (Monogenea: Gyrodactylidae): ultrastructure of basal matrices, cytoplasmic membranes and the birth plug, and comparison with other reproductive epithelia. *Int. J. Parasitol.* 28: 1805-1815.

Kearn, G.C., 1998. *Parasitism and the Platyhelminthes*. (ed. Chapmann and Hall), London, pp. 104-112.

Kennedy, C.E.J., Endler, J.A., Poyton, S.L. and McMinn, H., 1987. Parasite load predicts mate choice in guppies (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* 21: 291-295.

Khan, R.A. and Kiceniuk, J.W., 1988. Effect of petroleum aromatic hydrocarbons on monogeneids parasitizing Atlantic cod, *Gadus morhua* L. *Bull. Environ. Contam. Toxicol.* 41: 94-100.

Khan, R.A. and Thulin, J., 1991. Influence of pollution on parasites of aquatic animals. *Adv. Parasitol.* 30: 201-238.

Khunyakari, R.P., Tare, V. and Sharma, R.N., 2001. Effects of some trace heavy metals on *Poecilia reticulata* (Peters). *J. Environ. Biol.* 22: 141-144.

Köck, G. and Bucher, F., 1997. Accumulation of zinc in rainbow trout (*Oncorhynchus mykiss*) after waterborne and dietary exposure. *Bull. Environ. Contam. Toxicol.* 58: 305-310.

Lester, R.J.G. and Adams, J.R., 1974. *Gyrodactylus alexandri*: reproduction, mortality, and effect on its host *Gasterosteus aculeatus. Can. J. Zool.* 52: 827-33.

Lindenstrøm, T. and Buchman, K., 2000. Acquired resistance in rainbow trout against *Gyrodactylus derjavini. J. Helmintol.* 74: 155-60.

Lopez, S., 1999. Parasitised female guppies do not prefer showy males. *Anim. Behav.* 57: 1129-1134.

Mackenzie, K., Wiliams, H.H., Wiliams, B., McVicar, A.H., and Siddall, R., 1995. Parasites as indicators of water quality and the potential use of helminth transmission in marine pollution studies. *Adv. Parasitol.* 35: 85-144.

Malmberg, G., 1990. On the ontogeny of the haptor and the evolution of the Monogenea. *Syst. Parasitol.* 17: 1-165.

McGeer, J.C., Szebedinszky, C., McDonald, D.G. and Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout.

1. Iono-regulatory disturbance and metabolic costs. *Aquat. Toxicol.* 50: 231-234.

Morley, N.J., Crane, M. and Lewis, J.W., 2001a. Toxicity of cadmium and zinc to miracidia of *Schistosoma mansoni*. *Parasitology* 122: 81-85.

Morley, N.J., Crane, M. and Lewis, J.W., 2001b. Toxicity of cadmium and zinc to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. *Int. J. Parasitol.* 31: 1211-1217.

Morley, N.J., Crane, M. and Lewis, J.W., 2002. Toxicity of cadmium and zinc mixtures to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. *Arch. of Environ. Contam. Toxicol.* 43: 28-33.

Morley, N.J., Crane, M. and Lewis, J.W., 2003a. Toxicity of cadmium and zinc to the cercarial activity of *Diplostomum spathaceum* (Tremadoda: Diplostomidae). *Folia Parasitologica* 50: 57-60.

Morley, N.J., Crane, M. and Lewis, J.W., 2003b. Effects of cadmium and zinc toxicity on orientation behavior of *Echinoparyphium recurvatum* (Trematoda: Echinostomidae) cercariae. *Dis. Aquat. Org.* 56: 89-92.

Nakano, T., Ono, K. and Takeuchi, M., 1992. Levels of zinc, irons, and copper in the skin of abnormally pigmented Japanese flounder. *Bull. Jpn. Soc. Sci. Fish.* 58: 2207.

Olafsdottir, S.H. and Buchmann, K., 2004. Dexamethasone treatment affects skin mucous cell density in *Gyrodactylus derjavini* infected *Salmo salar*. *J Helminthol.* 78: 87-90.

Olstad, K., Cable, J., Robertsen, G. and Bakke, T., 2006. Unpredicted transmission strategy of *Gyrodactylus salaris* (Monogenea: Gyrodactylidae): survival and infectivity of parasites on dead hosts. *Parasitology* 133: 33-41.

Pettersen, R.A., Vollestad, L.A., Flodmark, L.E., Poléo, A.B.S., 2006. Effects of aqueous aluminium on four fish ectoparasites. *Sci. Total Environ.* 369: 129-138.

Pickering, A.D. and Richards, R.H., 1980. Factors influencing the structure, function and biota of the salmonid epidermis. *Proc. Royal Soc. Edinburgh* 79B: 93-104.

Poléo, A.B.S., Schjolden, J., Hansen, H., Bakke, T.A., Mo, T.A., Rosseland, B.O., Lydersen, E., 2004. The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology* 128: 169-177.

Powell, J.J., Jugdaohsingh, R. and Thompson, R.P.H., 1999. The regulation of mineral absorption in the gastrointestinal tract. *Proc. Nutr. Soc.* 58: 147-153.

Reed, P., Francis-Floyd, R. and Klinger, R.E., 1998. *Monogenean Trematodes. FA-28*, Dep. Fisheries and Aquatic Sciences, Florida Cooperative Extension Service, Institute of Food and Agricultural Science, University of Florida.

Richards, G.R. and Chubb, J.C., 1996. Host response to initial and challenge infections, following treatment of *Gyrodactylus bullatarudis* and *Gyrodactylus turnbulii (Monogenea)* on the guppy *(Poecilia reticulata). Parasitol. Res.* 82: 242-247.

Richards, G.R. and Chubb, J.C., 1998. Long-term population dynamics of *Gyrodactylus bullatarudis* and *G. turnbulli* (Monogenea) on adult guppies (*Poecilia reticulata*) in 50-l experimental arenas. *Parasitol Res.* 84: 753-756.

Roberts, R.J. and Bullock, A.M., 1980. The skin surface ecosystem of teleost fishes. *Proc. Royal Soc. Edinburgh* 79B: 87-91.

Roche, H. and Boge, G., 1993. Effects of Cu, Zn, and Cr on antioxidant enzyme activities *in vitro* of red blood cells of a *Dicentrarchus labrax. Toxicol. In Vitro.* 7: 623-629.

Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat. Toxicol.* 22: 81-114.

Rombout, J.H.W.M., Taverne, N., van de Kamp, M. and Taverne-Thiele, A.J., 1993. Differences in mucus and serum immunoglobulins of carp (*Cyprinus carpio* L.). *Dev.and Compar. Immunol.* 17: 309-317.

Rougier, F., Troutaud, A., Ndoye, A. and Deschaux, P., 1994. Non-specific immune response of zebrafish, *Brachydanio rerio* (Hamilton-Buchanan) following copper and zinc exposure. *Fish Shellfish Immunol.* 4: 115-127.

Rozan, T.F., Lassman, M.E., Ridge, D.P. and Luther, G.W., 2000. Evidence for iron, copper and zinc complexation as multinuclear sulphide clusters in oxidic rivers. *Nature*. 406: 879-882.

Sanchez-Dardon, J., Voccia, I., Hontela, A., Chilmonczyk, S., Dunier, M., Boermans, H., Blakley, B. and Fournier, M., 1999. Immunomodulation by heavy metals tested individually or in mixtures in rainbow trout (*Oncorhynchus mykiss*) exposed *in vivo*. *Environ. Toxicol. Chem.* 18: 1492–1497.

Sauer, G.R. and Watabe, N., 1984. Zinc uptake and its effect on calcification in the scales of the mummichog, *Fundulus heteroclitus*. *Aquat. Toxicol.* 5: 51-66.

Sauer, G.R. and Watabe, N., 1989. Ultrastructural and histochemical aspects of zinc accumulation by fish scales. *Tissue and Cell* 21: 935-943.

Schliwa, M., 1975. Cytoarchitecture of surface layer cells of the teleost epidermis. *J. Ultrastruct. Res.* 52:377-386.

Schwerdtfeger, W.K., 1978. The structure of teleost epidermis with special reference to new qualitative and quantitative data from the guppy, *Poecilia reticulate* Peters. *Z. Mikrosk.-Anat. Forsch., Leipzig* 92: 193-205.

Scott, M.E., 1982. Reproductive potential of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* 85: 217-236.

Scott, M.E., 1985. Dynamics of challenge infections of *Gyrodactylus bullatarudis* Turnbull (Monogenea) on guppies, *Poecilia reticulata* (Peters). *J. Fish Dis.* 8: 495-503.

Scott, M.E. and Anderson, R.M., 1984. The population dynamics of *Gyrodactylus bullatarudis* (Monogenea) within laboratory populations of the fish host *Poecilia reticulata*. *Parasitology* 89: 159-194.

Scott, M.E. and Nokes, D.J., 1984. Temperature-dependent reproduction and survival of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* 89: 221-227.

Scott, M.E. and Robinson, M.A., 1984. Challenge infections of *Gyrodactylus bullatarudis* (Monogenea) on guppies, *Poecilia reticulata* (Peters), following treatment. *J. Fish. Biol.* 24: 581-586.

Sengel, P., 1986. Epidermal-dermal interactions. In *Biology of the Integument. 2. Vertebrates* (ed. Bereiter-Hahn J., Matoltsy A.G. and Richards K.S.), pp. 374-408. Berlin, Springer-Verlag.

Shears, M.A. and Fletcher, G.L., 1983. Regulation of $Zn^{2+}$ uptake from the gastrointestinal tract of a marine teleost, the winter flounder (*Pseudopleuronectes americanus*). *Can. J. Fish Aquat. Sci.* 40: S197-S205.

Shephard, K.L., 1994. Functions for fish mucus. *Rev Fish Biol. Fish.* 4: 401-429.

Shinn, A.P., Gibson, D.I. and Sommerville, C., 1993. An SEM study of the haptoral sclerites of the genus *Gyrodactylus* Nordmann, 1832

(Monogenea) following extraction by digestion and sonication techniques. *Syst. Parasitol.* 25: 135–144.

Shinn, A.P., Sommerville, C. and Gibson, D.I., 1997. Argentophilic structures as a diagnostic criterion for the discrimination of species of the genus *Gyrodactylus* von Normann (Monogenea). *Syst. Parasitol.* 37: 47-57.

Sigh, J. and Buchmann, K., 2000. Associations between epidermal thionin-positive cells and skin parasitic infections in brown trout *Salmo trutta. Dis. Aquat. Org.* 412(2): 135 –139.

Sinha, G.M., Chakravorty, P., 1982. Characterization and distribution of neutral and acidic mucins in the alimentary canal of an Indian freshwater major carp, *Catla catla* (Hamilton) by histochemical methods. *Gegenbaurs Morphol. Jahrb.* 128: 188-200.

Smyth, J.D. and Halton, D.W., 1983. *The physiology of trematodes.* (ed. Cambridge University Press), pp.7-8, 45.

Soleng, A. and Bakke, T.A., 1997. Salinity tolerance of *Gyrodactylus salaris* (Platyhelminthes, Monogenea): laboratory studies. *Can. J. Fish Aquat. Sci.* 54: 1837–1845.

Soleng, A., Bakke, T.A. and Hansen, L.P., 1998. Potential for dispersal of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) by sea-running stages of the Atlantic salmon (*Salmo salar*): field and laboratory studies. *Can. J. Fish. Aquat. Sci.* 55: 507–514.

Soleng, A., Poléo, A.B.S. and Bakke, T.A., 2005. Toxicity of aqueous aluminium to the ectoparasitic monogenean *Gyrodactylus salaris*. *Aquaculture* 250: 616–620.

Soleng, A., Poléo, A.B.S., Alstand, N.E.W. and Bakke, T.A., 1999. Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology* 119: 19-25.

St. Louis-Cormier, E.A., Osterland, C.K. and Anderson, P.D., 1984. Evidence for a cutaneous secretory immune system in rainbow trout (*Salmo gairdneri*). *Dev. Compar. Immunol.* 8: 71-80.

Steverding, D., Morgan, E., Tkaczynski, P., Walder, F., Tinsley, R., 2005. Effect of Australian tea tree oil on *Gyrodactylus* spp. infection of the three-spined stickleback *Gasterosteus aculeatus*. *Dis. Aquat. Organ.* 66: 29-32.

Sures, B., 2002. Competition for minerals between *Acanthocephalus lucii* and its definitive host perch (*Perca fluviatilis*). *Int. J. Parasitol.* 32: 1117 – 1122.

Turnbull, E.R., 1956. *Gyrodactylus bullatarudis* n.sp. from *Lebistes reticulates* Peters with a study of its life cycle. *Can.J.Zool.* 34: 583-594.

Vallee, B.L. and Falchuk, K.H, 1993. The biochemical basis of zinc physiology. *Physiol. Rev.* 73: 79-118.

Van Oosterhout, C., Joyce, D.A., Cummings, S.M., Blais, J., Barson, N.J., Ramnarine, I.W., Mohammed, R.S., Persad, N., Cable J., 2006 Balancing selection, random genetic drift, and genetic variation at the major

histocompatibility complex in two wild populations of guppies (*Poecilia reticulata*). *Evolution. Int. J. Org. Evolution.* 60: 2562-74.

Watanabe, T., Kiron, V. and Satoh, S., 1997. Trace minerals in fish nutrition. *Aquaculture* 151: 185-207.

Wekell, J.C., Shearer, K.D. and Houle, C.R., 1983. High zinc supplementation of rainbow trout diets. *Prog. Fish Cult.* 45: 144-147.

Wells, P.R. and Cone, D.K., 1990. Experimental studies on the effect of *Gyrodactylus colemanensis* and *G. salmonis* (Monogenea) on density of mucous cells in the epidermis of fry of *Oncorhynchus mykiss. J. Fish Biol.* 37: 599-603.

Whitear, M., 1986a. Epidermis. In *Biology of the Integument. 2. Vertebrates* (ed. Bereiter-Hahn J, Matoltsy AG and Richards KS), pp. 8-38. Berlin, Springer-Verlag.

Whitear, M., 1986b. Dermis. In *Biology of the Integument. 2. Vertebrates* (ed. Bereiter-Hahn J, Matoltsy AG and Richards KS), pp. 39-64. Berlin, Springer-Verlag.

Widianarko, B., Kuntoro, F.X.S., van Gestel, C.A.M., Verweij, R.A. and van Straalen, N.M., 2001. Toxicokinetics and toxicity of zinc under time-varying exposure in the guppy (*Poecilia reticulata*). *Environ. Toxicol. Chem.* 20: 763-768.

Widianarko, B., van Gestel, C.A.M., Verweij, R.A. and van Straalen, N.M., 2000. Associations between trace metals in sediment, water, and guppy, *Poecilia*

*reticulata* (Peters), from urban streams of Semarag, Indonesia. *Ecotoxicol. Environ. Saf.* 46B: 101-107.

Woodward, D.F., Brumbaugh, W.G., De Lonay, A.J., Little, E.E. and Smith, C.E., 1994. Effects on rainbow trout fry of a metals-contamined diet of benthic intervertebrates from the Clark Fork River, Montana. *Trans. Am. Fish. Soc.* 123: 51-62.

# CHAPTER 3

# CONCENTRATION-DEPENDENT EFFECTS OF WATERBORNE ZINC ON POPULATION DYNAMICS OF *GYRODACTYLUS TURNBULLI* (MONOGENEA) ON ISOLATED GUPPIES (*POECILIA RETICULATA*)[i]

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### 3.1 Summary

The effect of waterborne zinc (Zn) on *Gyrodactylus* population dynamics was studied on isolated guppies maintained at concentrations ranging from 0 to 240 µg Zn/l. After one week pre-exposure to Zn, each fish was experimentally infected with three gyrodactylids and parasite numbers were recorded daily on each fish until the fish either died or recovered from infection. Parasite establishment was most successful at 0 and 240 µg Zn/l (97%) compared with the intermediate Zn concentrations. Low to moderate concentrations of Zn were beneficial to the parasite, as evidenced by the concentration-dependent increase in peak parasite burden on recovered fish up to 120 µg Zn/l. In contrast, 240 µg Zn/l may have been toxic to the parasite, as both peak parasite burden (in fish that recovered from infection), and maximum rate of increase of the parasite population (in fish that died) declined at this concentration. The combined effect of infection and Zn is harmful to the fish, because mortality of infected fish (but not uninfected fish) increased with increasing Zn concentrations. We suggest that the observed mortality occurs because of the inability of fish to continuously produce mucous that is a key factor for protecting fish from both waterborne Zn and ectoparasites.

**Key Words:** *Gyrodactylus*, guppy, waterborne zinc, population dynamics, parasite, monogenean, heavy metals.

### 3.2 Introduction

Recent reviews (Poulin, 1992, Overstreet, 1993; Khan and Thulin, 1991) indicate that pollutants in the surrounding macroenvironment directly influence the population dynamics, distribution and dispersal of fish ectoparasites, often leading to increased ectoparasitism (Khan and Thulin, 1991; Poulin, 1992), especially in situations such as aquaculture where the high densities of fish provide an ideal environment for direct fish-to-fish transmission (Möller, 1987). Metal contaminants of the aquatic environment such as zinc (Zn) influence host-parasite interactions either through harmful effects to the parasite (Overstreet, 1993; Soleng *et al.,* 1999; Poléo *et al.,* 2003) and/or through reduced host resistance to infection (Khan and Thulin, 1991; Poulin, 1992; Overstreet, 1997). In turn, parasites may reduce host tolerance to pollutants (Khan and Thulin, 1991; Overstreet, 1997).

In aquatic systems, most Zn is found in the sediment. In the water column, although Zn is almost entirely particulate and coupled with dissolved organic and inorganic compounds (Florence, Morrison and Stauber, 1992; Rozan *et al.*, 2000), it may still be bioavailable (Gagnon and Saulnier, 2003). As an indispensable micronutrient, Zn is added as a growth-enhancing nutritional supplement in aquaculture (15 to 150 µg Zn/g in trout diet, Handy, 1996), but high quantities of unconsumed fish food result in Zn concentrations that exceed the Canadian Water Quality Guidelines (2001) of 30 µg Zn/l for the protection of aquatic life. In freshwater, Zn occurs at low concentrations (up to 20 µg/l) due to natural weathering of mineral deposits, but in industrialized areas waterborne Zn

concentrations can increase far above 100 µg/l (Handy, 1996). Seawater collected from the open ocean contains low levels of total Zn (0.01-0.6 µg/l), but Zn concentrations in coastal waters polluted with industrial effluents have been reported to reach 30,000 µg/l (Handy, 1996).

Excessive environmental Zn can seriously affect the survival of aquatic organisms such as fish (Widianarko *et al.*, 2000, 2001). Although most estimates of the 96 h $LC_{50}$ for fish are 1-10 mg Zn/l in soft water and 3-20 mg Zn/l in hard water, values range from 0.09 to 40 mg/l (Hogstrand and Wood, 1996). To date, toxicological studies on parasites have been performed on survival, activity, and encystment of larval digeneans, and results have varied depending on concentration, and the parasite species. Survival of miracidia and cercariae begins to be impaired at concentrations in the order of 100 µg/l (Asch and Dresden, 1977; Evans, 1982a, b: Morley, Crane and Lewis 2001a, 2001b, 2002). Exposure of Atlantic salmon to 50 to 400 µg Zn/l induced a significant decrease in the numbers of the ectoparasitic monogenean, *Gyrodactylus salaris* but had no apparent effect on the salmon (Poléo *et al.*, 2003). Thus, it appears that gyrodactylids are more sensitive to Zn than their hosts, but very few studies have examined the concurrent effects of pollutants and parasites on fish.

The aim of the current research was to further explore the effects of waterborne Zn on the population dynamics of gyrodactylids on isolated fish, using *Gyrodactylus turnbulli* Harris, 1986 that lives on the skin and fins of the guppy, *Poecilia reticulata* (Peters) as our experimental model. Gyrodactylids are small (0.4-0.8 mm body length) epidermal feeders infecting many families of marine and freshwater teleost fish (Kearn, 1998; Cone, 1999). Their viviparous

reproduction together with their direct fish-to-fish transmission makes them important pathogens in aquaculture (Cable, Harris and Tinsley, 1996; Cable et al., 2002a; Cable, Tinsley and Harris, 2002b; Johnsen and Jensen, 1991; Bakke *et al.*, 1992) where they cause both direct mortality and indirect mortality through secondary bacterial or fungal infections (Kearn, 1998; Woo, 1999). On isolated fish, gyrodactylid numbers increase exponentially after an initial lag period. Some fish are able to control the infection and parasite numbers subsequently decline to zero. Other fish succumb to the infection and die (Scott, 1985). The phases of infection (initial lag, time to peak, time to recovery) have highly variable lengths depending on fish response (Scott and Robinson, 1984; Scott, 1985; Richards and Chubb, 1996, 1998), parasite virulence and the influence of various environmental factors (Scott and Nokes, 1984; Soleng *et al.,* 1999; Poléo *et al.,* 2003). Our study was designed to determine whether the phases of infection are influenced by the concentration of waterborne Zn.

## 3.3 Material and Methods

### *3.3.1 Host and parasite*

The experiments were completed on isolated immature guppies (2 cm body length), naïve to *Gyrodactylus* infection, bred in our lab from a stock of adult guppies purchased through a local pet supplier. The fish were kept in transparent, covered, small plastic containers in an incubator at 25°C and constant light cycling (16L:8D), in 200 ml of artificial freshwater (Singh and Srinivastav, 1993) containing 0.123 g NaCl, 0.065 g $Na_2SO_4$, 0.004 g KCl, 0.117 g $CaCl_2$ and 0.04 g $MgCl_2$ per liter distilled water, and adjusted to pH 7.6 with

$NaHCO_3$ (Fisher Scientific, Montreal, Canada). Various concentrations of Zn (0, 15, 30, 60, 120, 240 µg/l) were added as $ZnCl_2$ with 98% purity (Anachemia Canada Inc., Montreal, Canada).

To confirm that Zn concentrations remained stable in the presence of fish that were fed Nutrafin Max Complete Flake Food (Rolf C. Hagen Inc., Montreal, Canada) once a day, we monitored Zn concentrations in our 200 ml containers at 0, 24 and 48 hours. For each of the six Zn concentrations at each of the three time points, a 50 ml sample from each of three replicate 200 ml containers was filtered, acidified to a pH of 2, and stored at 4°C. Samples were analyzed using inductively coupled plasma emission source in the chemistry laboratory of St. Lawrence Centre, Environment Canada, Montreal, Canada. Within each prepared concentration, there was no significant difference in Zn concentration over the 48 h period (Table 3.1). Based on these results, artificial freshwater solutions were used for all experiments, they were replaced every two days, and the experimental fish were fed Nutrafin Max Complete Flake Food flakes once a day. As it was not possible to prepare artificial freshwater that contained less than 8 µg Zn/l, all measured concentrations were approximately 8 µg/l higher than the intended values (Table 3.1). The detected concentration of 8.31 µg/l in solutions to which no Zn was added was similar to the average obtained from our guppy breeding tanks (8.6 ± 1.3 µg Zn/l). For convenience, we will refer to the amount of Zn added, rather than to the measured final concentrations.

Table 3.1: Measured Zn concentration in experimental solutions. Resulting Zn concentrations, averaged from samples collected at 0, 24 and 48 hours after isolated guppies were introduced into 200 ml of artificial freshwater to which various concentrations of Zn had been added. Fish were fed once at 24 hours.

| **Intended µg Zn / l** | **0** | **15** | **30** | **60** | **120** | **240** |
|---|---|---|---|---|---|---|
| **Measured µg Zn /l ± SE** | 8. 3 ± 0.7 | 23.7 ± 1.3 | 38.1 ± 2.1 | 68.5 ± 4.8 | 129.5 ± 8.6 | 260.3 ± 10.9 |

*Gyrodactylus turnbulli* was originally obtained from infected guppies purchased from a local pet supplier, and was maintained in the laboratory by weekly addition of naïve fish into tanks with infected guppies. Experimental fish were anaesthetized for a maximum of five minutes in 50 ml of 0.02% tricaine methanesulfonate (Finquel MS222, Argent Chemical Laboratories, Washington) buffered to a neutral pH with $NaHCO_2$, during infection and subsequent daily monitoring, using a dissecting microscope with a cold light source. Each experimental fish was infected by transfer of three *G. turnbulli* on a scale or small piece of fin from an infected donor to the caudal peduncle of a naïve recipient (Scott, 1982). All procedures were approved by a McGill University Animal Care Committee, in accordance with the Canadian Council on Animal Care Guidelines (1993).

*3.3.2 Experimental protocol and outcome measures*

Between 30 and 36 fish were randomly assigned to one of six solutions (0, 15, 30, 60, 120, 240 µg Zn/l). After one week pre-exposure to the respective Zn concentration, each fish was experimentally infected and then maintained in the same concentration of Zn until its death or until parasite numbers remained at zero for at least three consecutive days (defined as recovery). The number of parasites on each fish was recorded daily to follow the parasite population dynamics. Establishment (parasite numbers increased to at least 4 parasites within the first 5 days) was recorded for each of the Zn concentrations and a minimum for 24 infected fish per concentration. We recorded the following outcomes: (1) peak burden, (2) time to peak burden, (3) percent mortality of fish, (4) survival time for fish that died, (5) duration of infection on recovered fish, (6) percent of recovered fish with a long recovery period where parasite numbers remained low for more than five days after the rapid decline from peak burden, and (7) maximum intrinsic daily rate of increase of parasite population per fish, calculated as $\ln (N_2/N_1)$, where $N_1$ and $N_2$ are the parasite numbers on two consecutive days.

An additional 10 uninfected fish were maintained under identical conditions in each of the six Zn concentrations, and handled daily. Their survival was monitored for 45 days in order to determine whether fish mortality could be attributed to Zn exposure alone.

*3.3.3 Statistical analysis*

All categorical variables were analyzed using $\chi^2$ and the binomial 95% confidence limits for percentages are reported (Rohlf and Sokal, 1981). For all continuous variables, the mean and SE are reported. In order to determine whether the pattern of parasite burdens over time on individual fish differed among Zn concentrations, a repeated measures ANOVA including Zn and time as the independent variables was used. The effect of Zn concentration was assessed using Kruskal-Wallis non-parametric ANOVA for peak burden, time to peak, survival time and time to recovery. In addition, linear and polynomial regression analyses were used to detect trends in outcome parameters with increasing Zn concentration. The maximum intrinsic daily rate of increase of the parasite population was compared among Zn concentrations using a 1-way ANOVA. Analyses were performed using SAS Version 9.1 software. The level of significance was established at $p<0.05$; statistics are reported only for significant effects.

## 3.4 Results

Of the 197 fish that were exposed to *G. turnbulli*, 34 fish did not retain their infection for more than 4 days, and therefore the parasite was not considered to have established on these fish. The overall number of fish on which the parasite successfully established differed significantly among waterborne Zn concentrations ($\chi^2 = 14.19$, df = 5, $p = 0.0144$). Visual inspection of the data revealed that establishment was highest (97%) in fish exposed to 0 and 240 µg Zn/l and lowest (69%) in fish exposed to 15 µg Zn/l (Fig. 3.1).

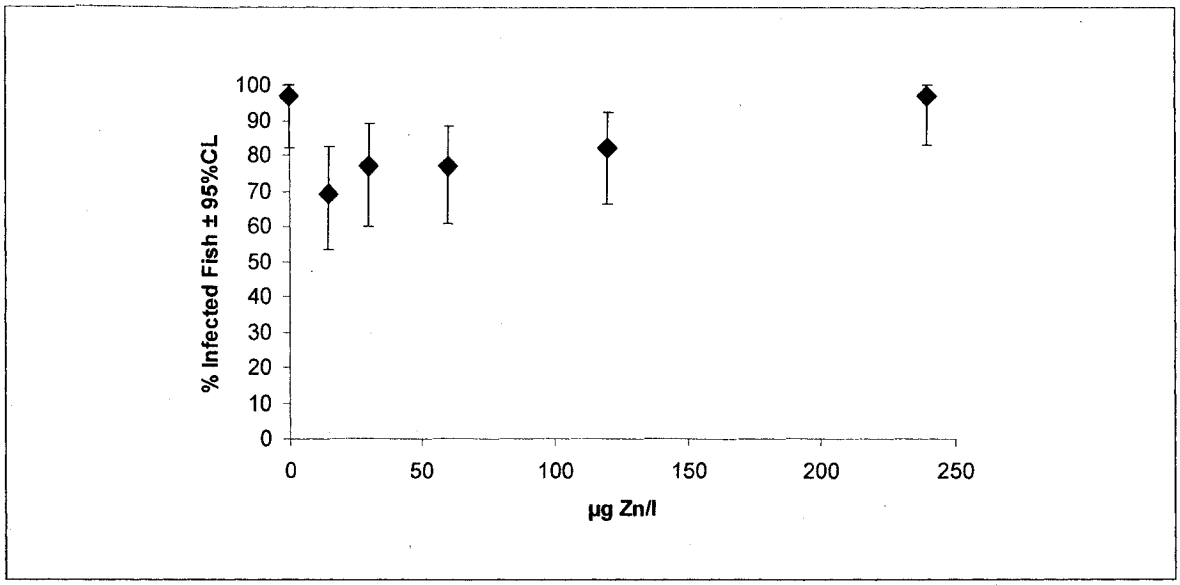
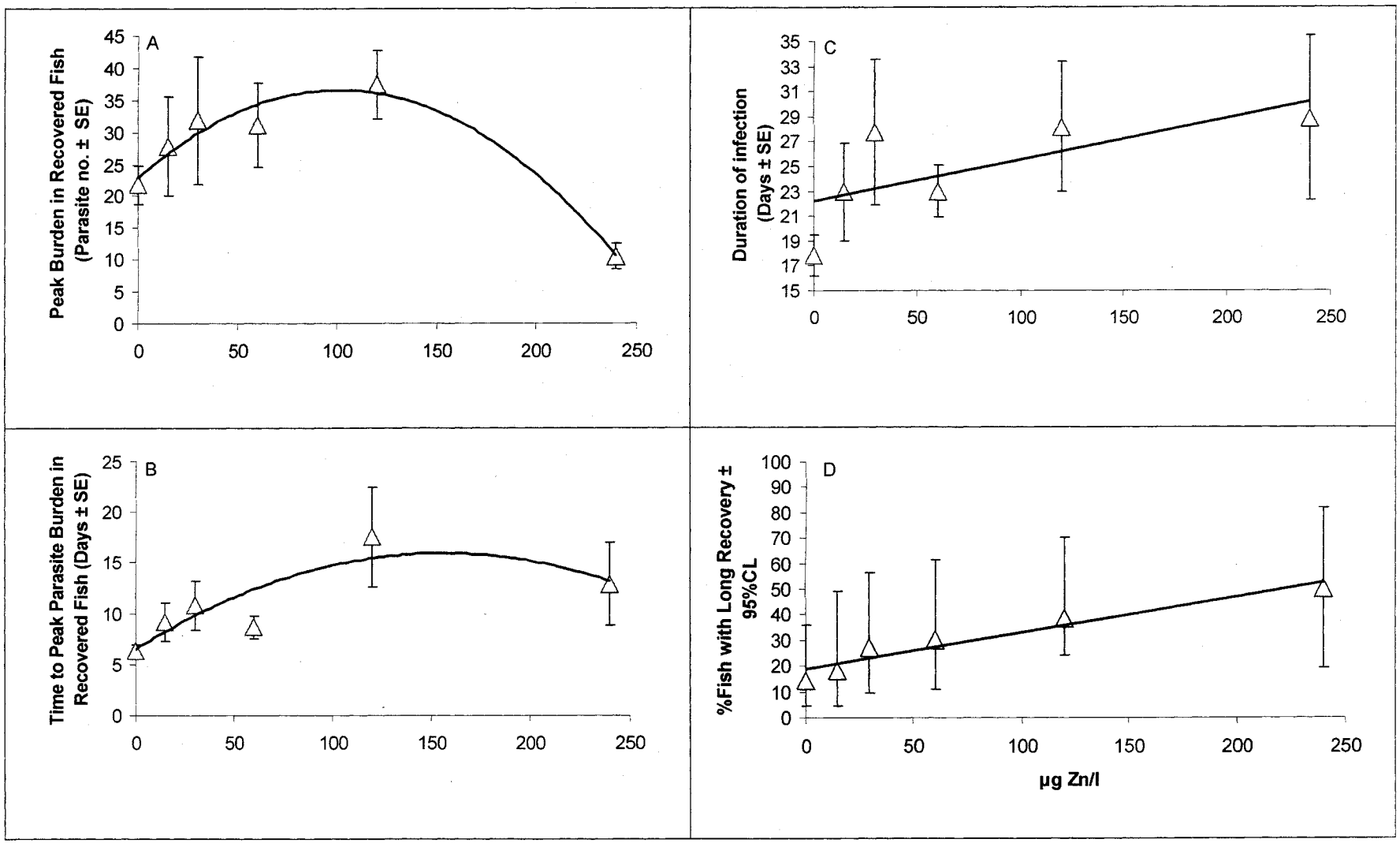



Fig. 3.1 The effects of waterborne Zn concentration on *Gyrodactylus turnbulli* establishment (♦) (n = 30, 36, 31, 35, 34, 31, respectively for concentrations ranging from 0 to 240 µg Zn/l). Isolated guppies were pre-exposed to various Zn concentrations for one week prior to infection with three parasites each.

*3.4.1 Population dynamics on infected fish that recovered*

A total of 69 fish became infected but then recovered. The average peak burden on these fish ranged from 10±2 to 37±5 parasites (Fig. 3.2A), and infections persisted between 18±2 and 29±7 days on average (Fig. 3.2C). Parasite numbers differed significantly with Zn concentration ($F_{5, 63} = 4.47$, $p = 0.015$) and with time ($F_{68, 1286} = 1.96$, $p < 0.0001$). In order to more fully explore these patterns, we compared the maximum intrinsic daily rate of increase of the parasite population, the peak parasite burden, the time to peak burden, the percentage of fish with a long recovery period and the duration of infection among Zn concentrations.

Fig.3.2 The effects of waterborne Zn concentration on infection parameters in guppies that recovered from an initial infection with three *Gyrodactylus turnbulli* (n = 22, 12, 11, 10, 8, 6, respectively for concentrations ranging from 0 to 240 µg Zn/l). Data (Δ) and best fit linear or polynomial regression (—); (A) peak parasite burden, $y = -0.0014 x^2 + 0.2761 x + 22.553$; (B) time to peak parasite burden, $y = -0.0004 x^2 + 0.116 x + 6.5098$; (C) duration of infection, $y = 0.0429 x + 21.005$; (D) percent of fish with long recovery phase, $y = 0.1406 x + 18.6$.


A
Peak Burden in Recovered Fish (Parasite no. ± SE)
0
5
10
15
20
25
30
35
40
45
0
50
100
150
200
250
C
Duration of infection (Days ± SE)
15
17
19
21
23
25
27
29
31
33
35
0
50
100
150
200
250
B
Time to Peak Parasite Burden in Recovered Fish (Days ± SE)
0
5
10
15
20
25
0
50
100
150
200
250
D
%Fish with Long Recovery ± 95%CL
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
µg Zn/l

Whereas the maximum intrinsic rate of the increase of the parasite population was unaffected by Zn concentration, both peak parasite burden (Fig. 3.2A; $F_{1, 68}$ = 3.80, p = 0.0274) and time to peak (Fig. 3.2B; $F_{1, 68}$ = 6.05, p = 0.0039) increased but then declined as Zn concentration increased and these two parameters were highly correlated ($r_{69}$ = 0.43, p = 0.0002). In contrast, the duration of the infection (Fig. 3.2C; $F_{1, 68}$ = 3.75, p = 0.0571 – borderline significance) and the proportion of fish with an extended recovery period (Fig 3.2D; $F_{1, 5}$ = 48.30, p = 0.0023) continued to increase linearly with increasing Zn concentration. Together these data indicate that Zn had positive effects on parasite population growth over concentrations up to 60 or 120 µg Zn/l and that even the highest concentration allowed prolonged infection of fish.

### *3.4.2 Effects of infection and Zn on guppy mortality*

The percent mortality among infected fish increased linearly ($F_{1, 5}$ = 8.26, p = 0.045) as Zn concentration increased, from 24% in fish maintained at 0 µg Zn/l to 80% for fish kept in 240 µg Zn/l (Fig. 3.3A). Repeated measures ANOVA of the pattern of parasite numbers over time revealed significant Zn, time and Zn*time effects ($F_{5, 88}$ = 5.21, p = 0.0003; $F_{48, 1508}$ = 9.40, p < 0.0001, and $F_{170, 1508}$ = 1.48, p < 0.0001 respectively). On average, the peak parasite burden in these fish ranged from 94 ±17 to 173±42 gyrodactylids per fish and the survival time ranged from 16±2 to 20±2 days, but neither parameter was significantly affected by Zn. The maximum intrinsic growth rate of the parasite population increased significantly as Zn concentration increased to 30 µg Zn/l, then declined (Fig. 3.3B; $F_{2, 93}$ = 3.76, p = 0.0271).

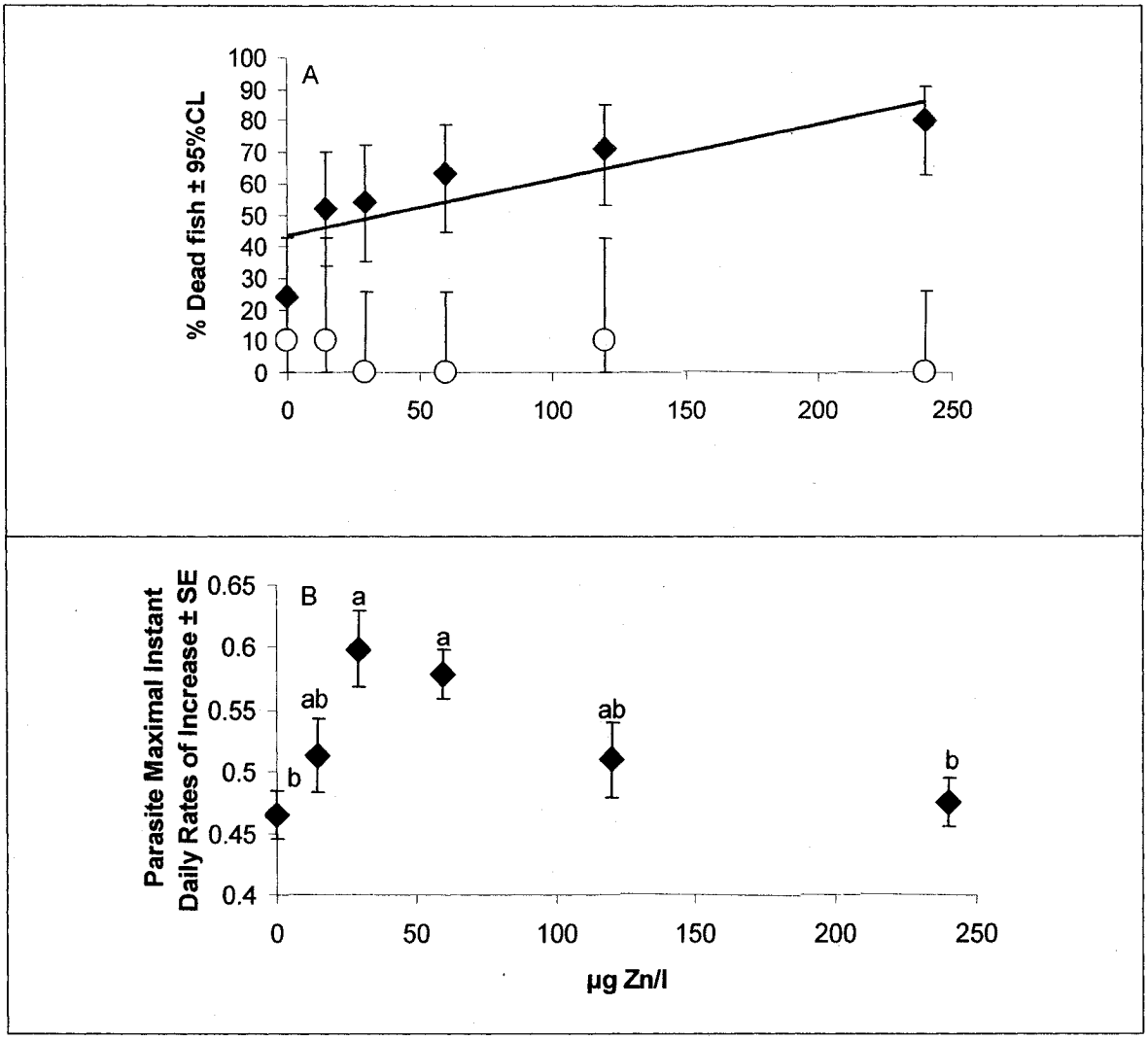



Fig. 3.3 The effects of waterborne Zn and *Gyrodactylus turnbulli* infection on (A) fish mortality among (○) uninfected fish monitored for 45 days (n = 10 / concentration) or (♦) fish infected with three *G. turnbulli* and then monitored until they recovered from infection or died (minimum of 24 fish per concentration); best fit linear regression for infected fish(—), $y = 0.1753x + 43.914$; and (B) maximum intrinsic rate of increase of the parasite on fish that died following infection (♦) (n = 7, 13, 13, 17, 20, 24, respectively). Means with the same letter are not significantly different.

In order to determine whether the observed mortality was due to infection, to Zn, or to the combined effects of the two stressors, we compared survival between fish exposed only to Zn, and those exposed to Zn and infection. Mortality was significantly higher in infected fish compared with uninfected fish exposed to waterborne Zn, especially at the higher Zn concentrations (Fig 3.3A; $\chi^2$ =26.25, df =11, p = 0.0355). Uninfected fish experienced no mortality for the first 35 days of exposure, regardless of Zn concentration, and mortality after day 35 was independent of Zn concentration (Fig. 3.3A).

We therefore concluded that the high mortality observed in infected fish was induced either by the parasite or by combined effects of parasite and Zn exposure, but not by Zn exposure alone.

### 3.5 Discussion

In this study, fish were broadly divided into three categories, based on their response to infection. On some fish, the parasite was unable to successfully establish an infection. The proportion of fish falling into this category was highest at intermediate zinc concentrations. Successful establishment of gyrodactylid infections depends in large part on the parasite finding a suitable epidermal habitat on the fish. For example, gyrodactylids avoid areas rich in mucous cells (Buchmann and Bresciani, 1998), and also get trapped and sloughed off in excess mucous (Wells and Cone, 1990). We observed patches of mucous over the guppy epidermis in fish that had been pre-exposed to between 15 and 60 µg Zn/l, and suggest that the presence of mucous would have made the epidermis a

less suitable habitat, thus explaining the reduced establishment success at intermediate concentrations of zinc.

The second category of fish were those on which the parasite successfully established and parasite numbers increased, but then declined to zero. Among these fish, the percentage of fish with a prolonged recovery period showed a linear increase in response to zinc concentration. One interpretation of these data is that the host was less able to control infection as zinc concentration increased, leading to longer recovery period. Interestingly, the most dominant host response to both gyrodactylid infection (Wells and Cone, 1990; Linderstrom and Buchmann, 2000) and to zinc exposure (Iger, Jenner and Wendelaar, 1994; Khunyakari, Tare and Sharma, 2001) is mucous production. Mucous and its associated complement and IL-1 are consistently reported as a requirement for killing of gyrodactylids (Buchmann, 1998, 1999; Harris, Soleng and Bakke, 1998; Buchmann and Bresciani, 1999). Mucous also greatly reduces underlying tissue exposure to zinc (Glover and Hogstrand, 2002) because of its high metal binding capacity (Handy, Eddy and Romain, 1989; Shephard, 1994), and this, together with the continual sloughing of both mucus and epithelial cells (Shephard, 1994; Glover and Hogstrand, 2002) prevents fish from accumulating zinc in tissues. Given that mucous production is central to control of infection and to protection against zinc, why then would fish be less able to control infection as zinc concentration increased? A variety of studies both on zinc (Iger *et al.*, 1994) and on gyrodactylid infection (Wells and Cone, 1990) show that fish cannot continually produce mucous. An initial stress induces mucous production but this can only be maintained for a short period of time, after which mucous release

stops until the mucous cells are repleted. This may help to explain the high establishment of the parasite at 240 µg Zn/l compared with intermediate zinc concentrations. In the case of more chronic stress, mucous production gradually declines, either because of exhaustion of mucous production capacity or because of a gradual acclimation to the stress which no longer induces a mucous production response (McGeer *et al.*, 2000). We suggest that the combined demands of high zinc exposure and infection exhausted the ability of the guppies to continue to produce mucous, leading to prolonged infection in those fish that were eventually able to recover.

Although there was no zinc-dependent effect of the maximum intrinsic growth rate of the parasite population in fish that recovered, the peak parasite load and the time to peak burden both increased then declined as zinc concentration increased. A depletion of mucous production would account for the increase in parasite numbers and time to peak at the intermediate zinc concentrations, but doesn't explain why these parameters would decline at the higher zinc concentrations. Zinc toxicity would account for this observation, and has been hypothesized by Poléo *et al.* (2003). They reported a significant decrease in numbers of *G. salaris* on Atlantic salmon exposed to Zn in concentrations ranging from 50 to 400 µg Zn/l. Zinc toxicity in invertebrates has also been indicated by reduced longevity of free-living miracidia and cercariae when exposed to 100 µg Zn /l and higher (Asch and Dresden, 1977; Evans, 1982a, b: Morley *et al.* 2001a, 2001b, 2002), and reduced reproduction in some terrestrial earthworms (Spurgeon and Hopkin 1996; Nursita, Singh and Lees,

2005). Our data from guppies exposed to high concentrations of zinc support the hypothesis that chronic exposure to zinc may be toxic to the parasite.

The third category of fish were those on which the infection became established and increased unchecked eventually leading to the death of the guppies. The proportion of infected fish that died increased with increasing zinc concentration. Under natural conditions, fish are exposed to many stressors including various pollutants and a range of infectious organisms. The ability of fish to survive these stressors depends not only on their tolerance to the pollutants, but also on their ability to kill pathogens or limit their growth, and to repair tissue damage induced by the pathogens. We observed very little mortality in our uninfected fish, suggesting that chronic exposure for 45 days to concentrations up to 240 µg Zn/l was insufficient to cause mortality in the absence of infection. This was not surprising as guppies are known to survive well in waters highly polluted with heavy metals (Widianarko *et al.* 2000, 2001), with $LC_{50}$ of 14.5±0.3 mg/l and 12.6±0.2 mg/l Zn after acute exposure for 24 and 48 h, respectively (Khunyakari *et al.* 2001). Whereas zinc alone had no impact on guppy survival, our study demonstrates that mortality on infected fish responded in a concentration-dependent manner to increasing concentrations of waterborne zinc. Gyrodactylids are known fish pathogens (Kearn, 1998; Cone, 1999, Scott, 1985) that browse the fish epidermis, ingesting both mucus and epidermal cells (Buchmann and Bresciani, 1998). Moreover, their opisthaptoral hooklets cause mechanical disruption of the epithelium that provides entry points for secondary bacterial infections (Cone, 1999). Scott (1985) reported 49% mortality among isolated guppies initially infected with three *G. turnbulli*, a value

comparable to what we observed among fish exposed to 15 µg Zn/l, but much lower than that observed at higher zinc concentrations.

The increased mortality at higher concentrations could have been an indirect result of increased pathology due to higher parasite loads as zinc concentration increased. However, this is not consistent with our observations. First, among the fish that died, the maximum intrinsic growth rate of the parasite population did not increase, but rather declined at the higher zinc concentrations. Second, the peak parasite burden on fish that died was independent of zinc concentration. Alternatively, the increased mortality at higher zinc concentrations might have resulted from impaired host responses at the higher concentrations. This would allow the parasite population to increase to high levels, unchecked by the host response, perhaps because of exhaustion of the ability to produce mucous. A third possibility is that tissue damage caused by the parasites may have facilitated entry of zinc into host tissues by breaking the mucous barrier, thus increasing its toxic effect on the guppy, and perhaps reducing their ability to repair tissue damage induced by infection.

In summary, we suggest that the demands on mucous production induced by the one-week pre-exposure to zinc prior to infection, together with subsequent infection-induced and zinc-induced production of mucous, exceeded the capacity of the host for sustained mucous release, allowing the parasite numbers to grow unchecked. Together, the pathogen and the pollutant reduced the percentage of fish that were able to survive the combined challenges in a zinc concentration-dependent manner. Although our data also suggest that chronic exposure to

high zinc concentrations may be toxic to the parasite, results indicate that zinc affects guppy-gyrodactylid interactions on isolated fish in a way that is, overall, more detrimental to the host than to the parasite.

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## References


Asch, H.L. and Dresden, M.H. (1977). *Schistosoma mansoni*: effects of zinc on cercarial and schistosomule viability. *Journal of Parasitology* 63, 80-86.

Bakke, T.A., Harris, P.D., Hansen, L.P. and Jansen, P.A. (1992). Host specificity and dispersal strategy in gyrodactylid monogeneans with particular reference to *Gyrodactylus salaris* (Platyhelminthes, Monogenea). *Diseases of Aquatic Organisms* 13, 45-57.

Buchmann, K. (1998). Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). *Diseases of Aquatic Organisms* 32, 195-200.

Buchmann, K. (1999). Immune mechanisms in fish skin against monogeneans – a model. *Folia Parasitologica* 46, 1-9.

Buchmann, K. and Bresciani, J. (1998). Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*: association between mucous cell density in skin and site selection. *Parasitology Research* 84, 17-24.

Buchmann, K. and Bresciani, J. (1999). Rainbow trout leukocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini. Diseases of Aquatic Organisms* 35, 13-22.

Cable, J., Harris, P.D. and Tinsley, R.C. (1996). Ultrastructural adaptations for viviparity in the female reproductive system of gyrodactylid monogeneans. *Tissue and Cell* 28, 515-526.

Cable, J., Scott, E.C.G., Tinsley, R.C. and Harris, P.D. (2002a). Behavior favoring transmission in the viviparous monogenean *Gyrodactylus turnbulli*. *Journal of Parasitology* 88, 183-184.

Cable, J., Tinsley, R.C. and Harris, P.D. (2002b). Survival, feeding and embryo development of *Gyrodactylus gasterostei* (Monogenea: Gyrodactylidae). *Parasitology* 124, 53-68.

Canadian Council of Ministers of the Environment, (2001). *Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life. http://www.ccme.ca/assets/pdf/e1_062.pdf*

Canadian Council on Animal Care. (1993). *Guide to the Care and Use of Experimental Animals*, Vol. 1, 2nd Ed., ed. E. D. Olfert, B. M. Cross, A. A. McWilliam.

Cone, D.K. (1999). Monogenea. In *Fish Diseases and Disorders. Vol. 1. Protozoan and Metazoan Infections* (ed. Woo, P.T.K.), pp 289-327. CABI Publishing, Wallingford, UK.

Evans, N.A. (1982a). Effect of copper and zinc upon the survival and infectivity of *Echinoparyphium recurvatum* cercariae. *Parasitology* 85, 295-303.

Evans, N.A. (1982b). Effect of copper and zinc on the life cycle of *Notocotyllus attenuatus* (Digenea: Notocotylidae). *International Journal for Parasitology* 12, 363-369.

Florence, T.M., Morrison, G.M. and Stauber, J.L. (1992). Determination of trace element speciation and the role of speciation in aquatic toxicity. *The Science of the Total Environment* 125, 1-13.

Gagnon, C. and Saulnier, I. (2003). Distribution and fate of metals in the dispersion plume of a major municipal effluent. *Environmental Pollution* 124, 47-55.

Glover, C.N. and Hogstrand, C. (2002). *In vivo* characterization of intestinal zinc uptake in freshwater rainbow trout. *Journal of Experimental Biology* 205, 141-150.

Handy, R.D. (1996). Dietary exposure to toxic metals in fish. In *Toxicology of Aquatic Pollution. Physiological, cellular and molecular approaches.* (ed. Taylor EW, Department of Biological Sciences, University of Birmingham), pp. 29-60. Cambridge University Press, Cambridge.

Handy, R.D., Eddy, F.B. and Romain, G. (1989). *In vitro* evidence for the ionoregulatory role of rainbow trout mucus in acid, acid/aluminum and zinc toxicity. *Journal of Fish Biology* 35, 737-747.

Harris, P.D., Soleng, A. and Bakke, T.A. (1998). Killing of *Gyrodactylus salaris* (*Platyhelminthes, Monogenea*) mediated by host complement. *Parasitology* 117, 137-143.

Hogstrand, C. and Wood, C.M., (1996). The physiology and toxicology of zinc in fish. In *Toxicology of Aquatic Pollution. Physiological, cellular and molecular approaches.* (ed. Taylor EW, Department of Biological Sciences, University of Birmingham), pp. 61-84. Cambridge University Press, Cambridge.

Iger, Y., Jenner, H. and Wendelaar Bonga, S.E. (1994). Cellular responses in the skin of rainbow trout (*Oncorhynchus mykiss*) exposed to Rhine water. *Journal of Fish Biology* 45, 1119-1132.

Johnsen, B.O. and Jensen, A.J. (1991). The *Gyrodactylus* story in Norway. *Aquaculture*. 98, 289-302.

Kearn, G.C. (1998). *Parasitism and the Platyhelminths*., London, ed. Chapmann and Hall, pp. 104-12.

Khan, R.A. and Thulin, J. (1991). Influence of pollution on parasites of aquatic animals. *Advances in Parasitology* 30, 201-38.

Khunyakari, R.P., Tare, V. and Sharma, R.N. (2001). Effects of some trace heavy metals on *Poecilia reticulata* (Peters). *Journal of Environmental Biology* 22, 141-144.

Lindenstrom, T. and Buchmann, K. (2000). Acquired resistance in rainbow trout against *Gyrodactylus derjavini. Journal of Helminthology* 74, 155-160.

McGeer, J.C., Szebedinszky, C., McDonald, D.G. and Wood, C.M. (2000). Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1. Iono-regulatory disturbance and metabolic costs. *Aquatic Toxicology* 50, 231-234.

Möller, H. (1987). Pollution and parasitism in the aquatic environment. *International Journal for Parasitology* 17, 353-361.

Morley, N.J., Crane, M. and Lewis, J.W. (2001a). Toxicity of cadmium and zinc to miracidia of *Schistosoma mansoni. Parasitology* 122, 81-85.

Morley, N.J., Crane, M. and Lewis, J.W. (2001b). Toxicity of cadmium and zinc to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. *International Journal for Parasitology* 31, 1211-1217.

Morley, N.J., Crane, M. and Lewis, J.W. (2002). Toxicity of cadmium and zinc mixtures to *Diplostomum spathaceum* (Trematoda: Diplostomidae)

cercarial survival. *Archives of Environmental Contamination and Toxicology* 43, 28-33.

Nursita, A.I., Singh, B. and Lees, E. (2005). The effects of cadmium, copper, lead, and zinc on the growth and reproduction of *Proisotoma minuta* Tullberg (Collembola). *Ecotoxicology and Environmental Safety* 60, 306-314.

Overstreet, R.M. (1993).Parasitic diseases of fishes and their relationship with toxicants and other environmental factors. In *Pathobioolgy of marine and estuarine organisms* (ed. Couch, J.A. and Fournie, W.), pp.111-156.CRC Press, Boca Raton, Florida.

Overstreet, R.M. (1997). Parasitological data as monitors of environmental health. *Parasitology* 39, 169-175.

Poléo, A.B.S., Schjolden, J., Hansen, H., Bakke, T.A., Mo, T.A., Rosseland, B.O., Lydersen, E. (2003). The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology* 128, 169-177.

Poulin, R. (1992). Toxic pollution and parasitism in freshwater fish. *Parasitology Today* 8, 58-61.

Richards, G.R. and Chubb, J.C. (1996). Host response to initial and challenge infections, following treatment of *Gyrodactylus bullatarudis* and *Gyrodactylus turnbulli* (Monogenea) on the guppy *(Poecilia reticulata). Parasitology Research* 82, 242-247.

Richards, G.R. and Chubb, J.C. (1998). Long-term population dynamics of *Gyrodactylus bullatarudis* and *G. turnbulli* (Monogenea) on adult guppies

(*Poecilia reticulata*) in 50-l experimental arenas. *Parasitology Research* 84, 753-756.

Rohlf, F.J. and Sokal, R.R. (1981). *Statistical Tables*, 2nd edition, 219 pp. W.H. Freeman and Company, NY.

Rozan, T.F., Lassman, M.E., Ridge, D.P. and Luther, G.W. (2000). Evidence for iron, copper and zinc complexation as multinuclear sulphide clusters in oxidic rivers. *Nature* 406, 879-882.

Scott, M.E. (1982). Reproductive potential of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* 85, 217-236.

Scott, M.E. (1985). Dynamics of challenge infections of *Gyrodactylus bullatarudis* Turnbull (Monogenea) on guppies, *Poecilia reticulata* (Peters). *Journal of Fish Diseases* 8, 495-503.

Scott, M.E. and Nokes, D.J. (1984). Temperature-dependent reproduction and survival of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* 89, 221-227.

Scott, M.E. and Robinson, M.A. (1984). Challenge infections of *Gyrodactylus bullatarudis* (Monogenea) on guppies, *Poecilia reticulata* (Peters), following treatment. *Journal of Fish Biology* 24, 581-586.

Shephard, K.L. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries* 4, 401-29.

Singh, S. and Srinivastav, A.K. (1993). Effects of calcitonin administration on serum calcium and inorganic phosphate levels of the fish, *Heteropneustes fossilis*, maintained either in artificial freshwater, calcium-rich freshwater,

or calcium-deficient freshwater. *The Journal of Experimental Zoology* 265, 35-39.

Soleng, A., Poléo, A.B.S., Alstand, N.E.W. and Bakke, T.A. (1999). Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology* 119, 19-25.

Spurgeon, D.J. and Hopkin, S. P. (1996). Effects of metal-contaminated soils on the growth, sexual development, and early cocoon production of the earthworm *Eisenia fetida*, with particular reference to zinc. *Ecotoxicology and Environmental Safety* 35, 86-95.

Wells, P.R. and Cone, D.K. (1990). Experimental studies on the effect of *Gyrodactylus colemanensis* and *G. salmonis* (Monogenea) on density of mucous cells in the epidermis of fry of *Oncorhynchus mykiss. Journal of Fish Biology* 37, 599-603.

Widianarko, B., Kuntoro, F.X.S., van Gestel, C.A.M., Verweij, R.A. and van Straalen, N.M. (2001). Toxicokinetics and toxicity of zinc under time-varying exposure in the guppy (*Poecilia reticulata*). *Environmental Toxicology and Chemistry.* 20, 763-768.

Widianarko, B., van Gestel, C.A.M., Verweij, R.A. and van Straalen, N.M., (2000). Associations between trace metals in sediment, water, and guppy, *Poecilia reticulata* (Peters), from urban streams of Semarag, Indonesia. *Ecotoxicology and Environmental Safety*, 46B, 101-107.

## CONNECTING STATEMENT BETWEEN CHAPTER 3 AND CHAPTER 4

Our first experiment revealed that after one week of pre-exposure to Zn, peak parasite burden and time to peak increased in recovered fish kept in up to 120 µg Zn/L, then declined, in the ones kept in 240 µg Zn/L (Gheorghiu *et al.*, 2006). We suggested that these patterns could be attributed either to a direct effect of Zn on parasites (beneficial for the concentrations up to 120 µg Zn/L, or toxic at 240 µg Zn/L), or an indirect effect of Zn on the parasite, mediated through the host response to both stressors.

The next manuscript describes a set of experiments designed to determine the impact of Zn on: (1) lifetime survival, reproduction and morphometrics of parasites on their host; (2) survival of detached parasites.

# CHAPTER 4

# EFFECTS OF WATERBORNE ZINC ON REPRODUCTION, SURVIVAL AND MORPHOMETRICS OF *GYRODACTYLUS TURNBULLI* (MONOGENEA) ON GUPPIES (*POECILIA RETICULATA*)[i]

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Permission to use the content of the manuscript in the present thesis is granted through the journal policies (appendix B).

### 4.1 Abstract

Recent reviews indicate that pollutants in the surrounding macroenvironment directly influence the population dynamics, distribution and dispersal of fish ectoparasites, often leading to increased parasitism. The aim of the current study was to explore the effects of sublethal concentrations of waterborne zinc (up to 240 µg Zn/L) on survival, reproduction and morphometrics of *Gyrodactylus turnbulli*, a viviparous monogenean infecting the skin and fins of the guppy, *Poecilia reticulata*. Parasite survival and reproduction on the fish were recorded daily for individual parasites maintained in isolated containers. Both survival and reproduction were reduced in 30 and 120 µg Zn/L, compared with 0, 15, and 60 µg Zn/L indicating direct toxic effects of Zn on the parasite. However, as generation time was unaffected by Zn, we attribute the reduced reproduction to the shorter lifespan. Parasite survival off the fish was monitored hourly. Average lifespan of the detached parasites decreased linearly from 19.5 h in 0 µg Zn/L to 17.3 h in 240 µg Zn/L, further supporting the direct toxic effect of Zn to the parasite. In addition, temporal dynamics of parasite morphometrics were monitored from mini-epidemics sampled after 1, 5, 10, and 15 days exposure to various Zn concentrations. All morphological parameters decreased significantly in response both to concentration and duration of exposure to waterborne Zn. Together these data clearly indicate that concentrations as low as 120 µg Zn/L are directly toxic to *G. turnbulli*.

*Keywords:* Heavy metal toxicity; Waterborne zinc; Guppy; Gyrodactylids; Reproduction; Lifespan; Survival; Morphometrics

## 4.2 Introduction

*Gyrodactylus* species are monogenean ectoparasites living on the skin, fins and gills of many families of marine and freshwater teleost fish (Harris *et al.*, 2004). There are over 400 species of *Gyrodactylus* species ranging between 0.2-0.8 mm in length, all with similar morphology (Bakke *et al.*, in press). The young newborn contains within its uterus several generations of embryos in sequential stages of development (Cable and Harris, 2002). The first daughter is probably an asexual clone of her mother, whereas subsequent daughters may be either sexually or parthenogenetically-derived (Cable and Harris, 2002). Newborn parasites attach to the fish, adjacent to their mother (Scott, 1982). Although dispersal occurs mainly during direct contact between fish, detached gyrodactylids can survive off the fish for several hours and are able to re-infect new hosts (Bakke *et al.*, 1992; Cable *et al.*, 2002a). Gyrodactylids ingest both epidermal cells and mucus (Buchmann and Lindenstrom, 2002), but monogeneans may supplement their diet with organic nutrients of low molecular weight that are absorbed directly from the water across the tegument (Smyth and Halton, 1983). Embryos within the uterus probably obtain nutrients across the uterine wall which is situated close to the parental gastrodermis (Cable *et al.*, 2002b).

As in other aquatic ectoparasites, environmental pollutants influence *Gyrodactylus* survival, reproduction and population dynamics in a concentration-dependent manner that is specific to the pollutant. For example, the prevalence and intensity of gyrodactylids increased in response to polyaromatic hydrocarbons (PAHs) (Khan and Kiceniuk, 1988). In contrast, aqueous aluminum

(50 - 200 µg/L) and zinc (50 - 400 µg/L) impaired *G. salaris* population growth (Soleng *et al.*, 1999; Poléo *et al.*, 2004), whereas neither copper (10 - 80 µg/L), iron (25 - 200 µg/L), nor manganese (100 - 800 µg/L) affected these parasites (Poléo *et al.*, 2004). Recently, we reported a concentration-dependent increase in mortality of guppies in response to the combined effects of aqueous zinc (Zn) and infection by *Gyrodactylus turnbulli* (Gheorghiu *et al.*, 2006). We further reported that among those fish that survived these combined stresses, peak parasite burden increased with increasing Zn concentration up to 120 µg/L, then declined. However, it was unclear whether parasites directly benefited from increased Zn concentration or whether elevated Zn impaired the host response, thus indirectly promoting parasite survival.

The present study was designed to determine whether waterborne Zn has direct effects on the survival, reproduction or morphometrics of *G. turnbulli,* a parasite on the skin and fins of the guppy, *Poecilia reticulata* (Peters). This experimental model is useful because guppies are easy to maintain and breed under laboratory conditions, and the parasite has a short generation time (days) and can be monitored without destructive sampling of the host. In the first experiment, we monitored lifetime survival and reproduction of individual parasites on isolated guppies (Experiment 1) where host response against the parasite was minimized by using fish with only 1 or 2 individual parasites. In Experiment 2, we examined the impact of waterborne Zn on the survival and reproduction of parasites that were detached from their fish host. Finally, in Experiment 3, we followed the temporal dynamics of parasite morphometrics in

small mini-epidemic tanks where parasite transmission among the fish ensured the persistence of the parasite (Scott and Anderson, 1984).

## 4.3 Materials and methods

### *4.3.1 General methods*

Experimental fish (standard body length 0.8-1.5 cm) were laboratory bred from a strain of feeder guppies obtained from a pet store in Montreal's West Island, and were naïve to gyrodactylids. All fish were kept at 25°C, in a 16 h light:8 h dark cycle, and were fed a Nutrafin Max Complete Flake diet once a day. *Gyrodactylus turnbulli* obtained from infected guppies purchased from the same local pet supplier was identified according to Harris *et al.* (1999). The strain was maintained in the laboratory by weekly addition of naïve fish into tanks with the infected guppies. A Nikon dissecting microscope equipped with a fiber optic light source was used for experimental infection and subsequent monitoring of fish anaesthetized for a maximum of 5 min in 50 ml of 0.02% tricaine methanesulfonate (Finquel MS222, Argent Chemical Laboratories, Washington) buffered to a neutral pH with $NaHCO_2$. All procedures were approved by a McGill University Animal Care Committee, in accordance with the Canadian Council on Animal Care Guidelines (2005).

Experimental zinc solutions were prepared as described by Gheorghiu *et al.* (2006). In brief, Zn was added to artificial freshwater to give concentrations of 0, 15, 30 (the maximal admissible limit for aquatic life according to Canadian Council of Ministers of the Environment, 2005), 60, 120 or 240 µg/L above the

baseline concentration of 8 µg/L. Solutions were changed every two days to ensure that zinc concentration in the water remained relatively constant.

*4.3.2. Experiment 1: Lifetime survival and reproduction of individual parasites*

In order to evaluate the concentration-dependent effects of Zn on parasite survival and reproduction, we followed individual F2 parasites from embryonic exposure to zinc, through their lifespan to death. Isolated guppies were kept in a transparent, covered plastic container in 200 ml of 0, 15, 30, 60 or 120 µg Zn/L (assigned at random). Each fish was initially infected with a single gravid specimen of *G. turnbulli,* and then monitored every morning. If visual inspection suggested that the parasite was likely to give birth within a few hours, the fish was re-examined again later the same day. Immediately after the initial parasite had given birth (F1 generation), the mother was removed and killed with fine forceps. Similarly, immediately after her daughter had given birth (F2 generation), the F1 daughter was killed. We defined day 0 as the day of birth of the F2 daughter, and this worm was monitored until its death. Each time the F2 daughter gave birth, its daughter was killed. We recorded the total number of daughters, the age of the F2 daughter at each birth and the lifespan of the F2 daughter (n = 13, 14, 19, 13, 16, respectively, for each concentration of Zn). Generation time was estimated as the age of the F2 daughter at the time of birth of her average daughter.

*4.3.3 Experiment 2: Survival and reproduction of detached parasites*

As detached parasites are able to re-infect fish, it was important to consider the influence of Zn concentration on parasites isolated from their host. This also enabled us to assess direct effects of Zn on the parasite, independent of host effects. For this experiment, we removed individual parasites from recently killed fish from the parasite culture, being careful to avoid contact between the parasite and any host body fluids. The parasites were individually transferred in 20 µL of artificial freshwater to the bottom of a 96 well microtitre plate (Costar Clear Polystyrene 96-Well Plates, Fisher Scientific, Montreal, Canada) containing 80 µL of artificial freshwater. To each well, 100 µl of Zn solution was added to give a final concentration of 0, 15, 30, 60, 120, or 240 µg Zn/L above baseline Zn concentration. Each parasite was examined one hour after transfer, and any that showed signs of damage or that were dead were discarded and excluded from the data set. Parasites were then examined every 2 h, and the timing and number of births as well as the time of death of both the initial parasite and any daughters were recorded. Non-motile parasites that did not respond to a gentle water current created by moving a needle were considered dead; if they responded to the water current they were classified as moribund. The lifespan of each parasite was recorded as well the percent of lifespan in a moribund state. This experiment was replicated five times to give a total of 48-56 parasites / concentration.

*4.3.4. Experiment 3: Temporal dynamics of morphometrics in parasite populations*

Given the critical role of Zn in cell division, growth and development (Eisler, 1993), we examined the potential effect of Zn on the size of parasites exposed for 5, 10 and 15 days to various concentrations of waterborne Zn. This experiment focused on soft body parts because we anticipated more rapid effects of Zn on these tissues rather than hooks and hamuli. Moreover our primary interest concerned parasite reproduction and survival rather than attachment and movement. A set of mini-epidemics was established by placing one infected guppy together with three uninfected guppies in 1 liter plastic tanks containing one of six waterborne Zn solutions (0, 15, 30, 60, 120 or 240 µg/L). When an infected fish died, it was replaced with an uninfected one to ensure maintenance of the parasite population for 15 days. On days 5, 10 and 15 post-infection (pi) at least one infected fish in each Zn concentration was killed in 50 ml of 0.03% MS222. Between 20 and 33 parasites / concentration were removed from the fish and transferred with 2 ml of MS222 into a well of a 6-well tissue culture plate. Aqueous neutral red vital stain (4 ml of 0.005% solution) was added to each well and the plate was kept at 4°C for 4-5 h to stop their movements and improve the reliability of measurements. Each parasite was then placed on a microscope slide under cover slip pressure. Total body length, body width at the widest part of the uterus, pharynx diameter, and opisthaptor length and width were measured using a Nikon compound microscope at x125 magnification. Body area was estimated as the product of body length and body width. For each Zn concentration and for each time point, measurements were made on parasites

from three replicate mini-epidemics. Moreover, to determine whether the addition of Zn in artificial freshwater induced any osmotic effect on parasite morphometrics, infected fish were kept individually in 200 ml of 0, 30, 120 or 240 µg Zn/L for 24 h before worms were measured as described above (n = 35 parasites / concentration).

*4.3.5. Statistical analysis*

The effect of Zn concentration on individual lifespan, number of births, the intervals between births, generation time and parasite morphology was assessed using $\chi^2$, Kruskal-Wallis non-parametric ANOVA, one- and two-way ANOVAs with Tukey's post-hoc test, depending on the parameter. Average lifespans were estimated by Probit analysis. Linear regression analysis was used to examine the relation between lifespan and number of offspring, and between zinc concentration and lifespan. The mean and SE as well as the binomial 95% confidence limits for percentages are reported (Rohlf and Sokal, 1981). Analyses were performed using SAS Version 9.1 software. The level of significance was established at $p<0.05$; statistics are reported only for significant effects.

**4.4 Results**

*4.4.1. Experiment 1: Lifetime survival and reproduction of individual parasites*

On average, the F2 daughters maintained in 0 µg Zn/L survived for 7.9 d and had 3.2 daughters, with a generation time of 3.6 d (Table 1). Poorer survival and reproduction occurred in parasites kept in 30 µg Zn/L; they survived only for

5.8 d and had 2.5 daughters, with an average generation time of 2.7 d. Between these two extremes of life history traits, parasites kept in 15 or 120 µg Zn/L had intermediate values of average lifespan, number of births and generation time. To explore this further, we classified parasite lifespan as short (1-4 d), medium (5-8 d) or long (9-12 d). Comparison across Zn concentrations confirmed that parasites survived longer in 0 µg Zn/L than in 30 or 120 µg Zn/L (Table 4.1), a difference driven by the very low percentage of parasites that survived for more than 9 d at the higher concentrations ($\chi^2_8$=16.72, $p<0.05$). Surprisingly, survival and reproduction of F2 parasites kept in 60 µg Zn/L were similar to parasites kept in 0 µg Zn/L (Table 4.1).

As with lifespan, the total number of births was lower at 30 and 120 µg Zn/L than at 0 µg Zn/L. At 30 and 120 µg Zn/L, low fecundity (0-2 daughters) was more common than higher fecundity (3-5 daughters) whereas the two categories were equally common in parasites maintained at 0 µg Zn/L (Table 4.1). The number of offspring increased linearly with lifespan ($p<0.0001$), such that longer lived parasites had more offspring and this was observed at each Zn concentration. Although generation time appeared to be lower for parasites kept at 30 µg Zn/L, neither the average generation time, nor the average age of the F2 daughter at birth of her first daughter nor the average inter-birth interval for subsequent births differed significantly among Zn concentrations (Table 4.1).

Table 4.1 Lifetime survival and reproduction of individual *Gyrodactylus turnbulli* on the fish in response to waterborne zinc (Zn).

| | µg Zn/L | | | | |
|---|---|---|---|---|---|
| | 0 | 15 | 30 | 60 | 120 |
| Average Lifespan (days) ± SE | 7.9 ± 0.9 | 6.9 ± 0.8 | 5.8 ± 0.6 | 7.6 ± 0.8 | 6.9 ± 0.6 |
| % with Lifespan Exceeding 8 Days | 62 | 43 | 10* | 46 | 12* |
| Average Number of Births ± SE | 3.2 ± 0.4 | 3.1 ± 0.4 | 2.5 ± 0.3 | 3.2 ± 0.4 | 2.9 ± 0.3 |
| % with 3 or More Births | 62 | 50 | 21* | 54 | 19* |
| Average Generation Time (days) ± SE | 3.6 ± 0.4 | 3.2 ± 0.4 | 2.7 ± 0.3 | 3.2 ± 0.4 | 3.0 ± 0.3 |
| Average Age at Birth of First Daughter (days) ± SE | 1.1 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.04 | 1.1 ± 0.1 | 1.0 ± 0.04 |
| Average Inter-Birth Interval (days) ± SE | 3.4 ± 0.2 | 3.3 ± 0.2 | 3.8 ± 0.2 | 3.2 ± 0.3 | 3.8 ± 0.2 |

* Significantly different from 0 µg Zn/L ($p < 0.025$

Together, these data demonstrate that parasite survival responded in a non-linear manner to increasing concentrations of Zn, with lowest values at 30 and 120 µg Zn/L and highest values at 0 µg Zn/L. Although parasite reproduction showed a similar pattern, the shorter lifespan of these parasites may explain the reduced reproduction.

*4.4.2. Experiment 2: Survival and reproduction of detached parasites*

Parasites that had been removed from the host had an estimated average lifespan ($LT_{50}$) of 19.4 h in 0 µg Zn/L. Lifespan decreased linearly ($F_{1,4}$=20.88, p=0.0103) with increasing concentrations of Zn to 17.3 h for parasites kept in 240 µg Zn/L (Fig. 4.1). Parasites not exposed to Zn were moribund for only 14.7% of their life off the fish whereas among parasites exposed to Zn, the percent of lifespan in a moribund state was similar among all concentrations (19.7%) and significantly higher for 120 µg Zn/L than for 0 µg Zn/L ($F_{5, 305}$ = 2.51, p = 0.0301). Approximately 12% of detached parasites gave birth or aborted their embryos, but given the low sample size, we were unable to draw conclusions about the impact of Zn on reproduction of detached parasites.

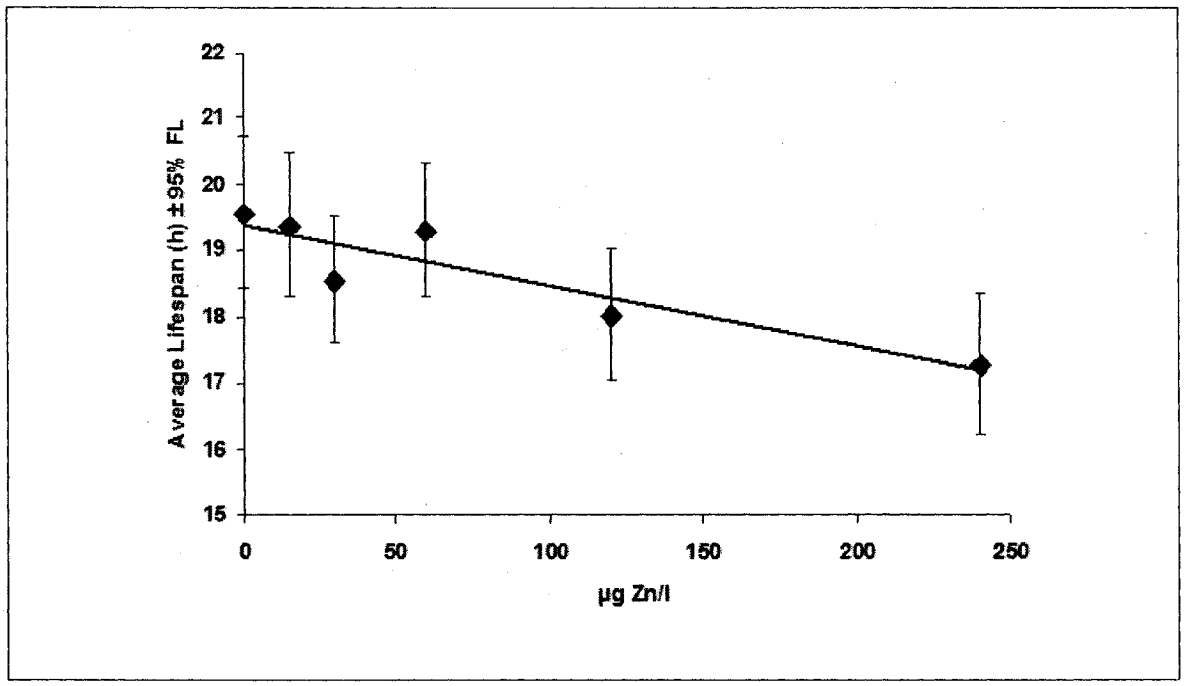


Fig. 4.1 Estimated average lifespan of detached *Gyrodactylus turnbulli* exposed to different waterborne zinc (Zn) concentrations. Solid line indicates best fit linear regression model (y = -0.0091x + 19.375).

*4.4.3. Experiment 3: Temporal dynamics of morphometrics in parasite populations*

For parasites kept at 0 µg Zn/L, none of the morphological measurements differed over time, whereas all morphological parameters were significantly affected by waterborne Zn, by duration of Zn exposure and by the interaction between the two factors (Table 4.2). In order to confirm that the effect of Zn was not due to acute osmotic stress on the parasite, we compared the size of parasites after 24 h exposure to 0, 30, 120 and 240 µg Zn/L (Fig. 4.2) and found no significant effect of Zn concentration on any of the morphometric parameters. After 5 d exposure, body area was markedly lower at all concentrations

compared with 0 µg Zn/L and effects were largely dose-dependent (Fig. 4.2). With prolonged exposure to Zn, the effect of Zn was more pronounced, with a concentration-dependent decrease detected both at 10 and 15 d exposure. Similar patterns were seen for all morphometric indicators (Table 4.2).

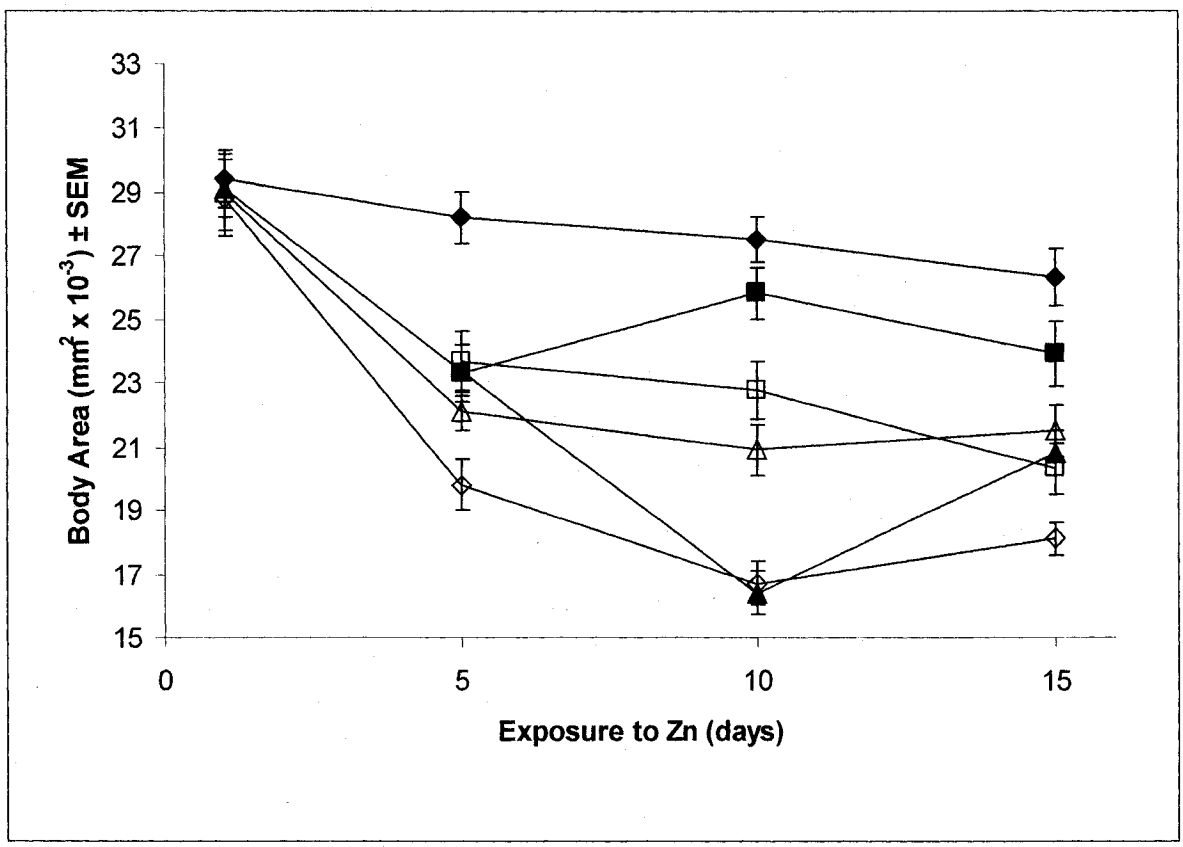


Fig. 4.2 Temporal dynamics of *Gyrodactylus turnbulli* morphometrics in response to different waterborne zinc (Zn) concentrations. ♦ - 0 µg Zn/L; ■ – 15 µg Zn/L; Δ - 30 µg Zn/L; □ – 60 µg Zn/L; ▲- 120 µg Zn/L; ◊ - 240 µg Zn/L.

### 4.5 Discussion

For gyrodactylids, as for all living organisms, Zn is presumably an essential structural, catalytic and regulatory micronutrient for many enzymes and critical for protein synthesis, cell proliferation, growth, development and

reproduction (Vallee and Falchuk, 1993; Hogstrand and Wood, 1996). On the other hand, high concentrations of Zn are likely to be toxic, as Zn is known to impair ion regulation (McGeer *et al.*, 2000) and cellular energy production (Dineley *et al.*, 2003), to cause apoptosis, and to be mutagenic and teratogenic (Eisler, 1993). The potential interactions between gyrodactylids and Zn are further complicated by the effects of Zn on the host. Fish regulate uptake of Zn across epithelial tissues by release of mucous that traps Zn, preventing its entry into tissues (Handy *et al.*, 1989; Shephard, 1994). Fish also release mucous as a defense mechanism against pathogens, including gyrodactylids (Lester and Adams, 1974; Buchmann and Lindenstrom, 2002). Thus, the mucous release induced by Zn may indirectly affect the parasite.

We previously reported that parasite population growth improved when *Gyrodactylus turnbulli* was maintained at Zn concentrations as high as 120 µg Zn/L and had speculated that parasite survival and/or reproduction might be directly enhanced as Zn concentration increased (Gheorghiu *et al.*, 2006). If true, our present study should have revealed prolonged parasite lifespan both on and off the host and/or increased rates of reproduction as Zn concentration increased up to 120 µg/L. However, this was not the case. In fact, both parasite survival and total number of offspring were significantly lower at 120 µg Zn/L than at 0 µg Zn/L. In addition, survival of detached parasites declined with increasing Zn concentrations, as did parasite size. Thus, it is clear that 120 µg/L Zn is toxic to this parasite. This is consistent with the reduced population growth of *Gyrodactylus salaris* on Atlantic salmon exposed to 50 to 200 µg Al/L or 50 to 400 µg Zn/L (Poléo *et al.*, 2004)

Table 4.2 Temporal dynamics of morphometrics of *Gyrodactylus turnbulli* populations in response to waterborne zinc (Zn).*

| | **Time Effects (Pooled across Zn Concentrations)** | | | | **Zn Effects (Pooled across Time)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | d1 | d5 | d10 | d15 | Zn0 | Zn15 | Zn30 | Zn60 | Zn120 | Zn240 |
| Body Length (µm) | $387^{a} \pm 5$ | $359^{b} \pm 4$ | $341^{c} \pm 3$ | $332^{c} \pm 3$ | $378^{A} \pm 4$ | $354^{B} \pm 5$ | $338^{C} \pm 4$ | $348^{BC} \pm 5$ | $340^{C} \pm 4$ | $312^{D} \pm 4$ |
| Body Width (µm) | $75.0^{a} \pm 0.9$ | $66.1^{b} \pm 0.6$ | $63.8^{c} \pm 0.6$ | $65.8^{b} \pm 0.6$ | $72.2^{A} \pm 0.6$ | $67.2^{B} \pm 0.8$ | $65.5^{BC} \pm 0.8$ | $62.6^{D} \pm 0.8$ | $63.5^{CD} \pm 0.9$ | $62.3^{D} \pm 0.8$ |
| Total Body Area ($mm^2 \times 10^{-3}$) | $29.1^{a} \pm 0.5$ | $24.1^{b} \pm 0.4$ | $22.2^{c} \pm 0.4$ | $22.2^{c} \pm 0.4$ | $27.6^{A} \pm 0.4$ | $24.3^{B} \pm 0.5$ | $22.5^{C} \pm 0.5$ | $22.2^{C} \pm 0.5$ | $21.8^{C} \pm 0.5$ | $19.8^{D} \pm 0.5$ |
| Pharynx diameter (µm) | $35.1^{a} \pm 0.4$ | $35.1^{a} \pm 0.3$ | $31.7^{b} \pm 0.3$ | $31.5^{b} \pm 0.3$ | $35.5^{A} \pm 0.3$ | $33.4^{B} \pm 0.4$ | $32.4^{CD} \pm 0.4$ | $33.1^{BC} \pm 0.4$ | $31.8^{D} \pm 0.4$ | $29.6^{E} \pm 0.4$ |
| Opisthaptor Length (µm) | $68.2^{a} \pm 0.8$ | $63.2^{b} \pm 0.6$ | $62.2^{bc} \pm 0.5$ | $61.4^{c} \pm 0.5$ | $67.7^{A} \pm 0.6$ | $61.8^{BC} \pm 0.8$ | $61.4^{BC} \pm 0.6$ | $61.9^{B} \pm 0.7$ | $61.9^{B} \pm 0.6$ | $60.1^{C} \pm 0.6$ |
| Opisthaptor Width (µm) | $90.9^{a} \pm 0.9$ | $84.4^{b} \pm 0.6$ | $82.4^{c} \pm 0.6$ | $80.6^{c} \pm 0.5$ | $88.9^{A} \pm 0.6$ | $85.6^{B} \pm 0.9$ | $81.6^{C} \pm 0.7$ | $79.8^{CD} \pm 0.7$ | $81.5^{C} \pm 0.8$ | $79.0^{D} \pm 0.8$ |

* Mean ± SE; lower case superscripts indicate significant differences over time; uppercase superscripts indicate significant differences among Zn concentrations. In all cases, the main effects of Time, Zinc and the Time*Zn interaction were highly significant ($P<0.0001$).

Our most direct measure of the specific effect of Zn on the parasite was the reduced survival of detached parasites that were exposed to Zn but not to host responses. This observation is consistent with reduced lifespan of digenean miracidia and cercariae exposed to Zn, in concentrations in the order of 100 µg/L (Asch and Dresden, 1977; Evans, 1982a, b: Morley *et al.*, 2001a, b, 2002). Although the decrease in average lifespan between 0 and 240 µg Zn/L was only about 2 h, we suspect that the ability to re-attach to a fish may have been more dramatically affected than survival as suggested by Olstad *et al.* (2006). Toxicity of Zn to detached parasites may be associated not only with increased apoptosis and impaired ion regulation, but also with energy balance. Detached gyrodactylids continue to give birth (personal observations) and need to maintain energy stores not only for reproduction but also for re-attachment to fish that may swim by. Furthermore, gyrodactylids, like digenean larvae, may sequester excessive metals in secretory structures associated with the tegumental surface as a means to detoxify Zn (Morley *et al.*, 2003) and this is likely an energy-demanding process. However, detached gyrodactylids no longer feed and even if they re-absorb nutrients from their *in utero* embryos (Cable and Tinsley, 1991; Cable and Harris, 2002), energy stores will decline more rapidly at higher Zn concentrations. In addition, Zn impairs cellular energy production in other organisms (Dineley *et al.*, 2005). Together the reduced energy stores and reduced energy production may contribute to the toxic effect on parasite survival. Not only was Zn toxic to detached parasites, but also it was toxic to parasites living on the fish. Parasite survival was reduced at Zn concentrations as low as 30 µg Zn/L, but interestingly parasites were more tolerant to 60 µg Zn/L than to

30 µg Zn/L. At 120 µg Zn/L, parasite survival was impaired to a similar extent as in parasites maintained at 30 µg Zn/L. While the reduced lifespan at 30 and 120 µg Zn/L could be explained by direct toxicity to the parasite, the better survival at 60 µg Zn/L was unexpected according to classic models of dose-dependence of toxicants (Calabrese and Baldwin, 2003). Rather, it is suggestive of a non-linear hormetic response (Calabrese and Baldwin, 2003) where the net effect of interacting factors results in a more complex pattern. In this system, the non-linear dose-response may result from the efforts of the fish to exclude entry of Zn into its tissues, combined with the direct toxic effects of Zn on the parasite. When exposed to waterborne toxicants, including Zn, fish immediately release mucous over epithelial surfaces to trap Zn and prevent its entry into the body (Handy *et al.,* 1989; Shephard, 1994; Hogstrand and Wood, 1996; Khunyakari, *et al.*, 2001), but are unable to sustain mucous release over prolonged periods of time (Eddy and Fraser, 1982), especially at higher concentrations. Thus, as Zn concentrations increases from 0 to 15 to 30 µg Zn/L, the release of higher amounts of mucous protects the host, but also removes parasites trapped in sloughed off sheets of mucus (Lester and Adams, 1974). When Zn concentrations exceed 30 µg Zn/L, the initial high rate of mucous production may not be sustainable (Eddy and Fraser, 1982), in which case the probability of parasites being trapped in mucous is reduced. This would account for the increased survival between 30 and 60 µg Zn/L. Concurrently, however, Zn exerts direct toxic effects on the parasite, which would be expected to increase as Zn concentration increases, thus explaining the reduced lifespan of the parasite between 60 and 120 µg Zn/L.

The concentration response of parasite reproduction to Zn was very similar to that of parasite lifespan, thus we attribute this result to the shortened lifespan of the parasite rather than to any direct toxic effect of Zn on parasite reproduction. It is not surprising that short-lived parasites have fewer offspring, but it is intriguing that Zn appears not to affect reproduction of gyrodactylids, given that the reproductive system is very sensitive to Zn in other organisms (Eisler, 1993). Perhaps the unique reproductive biology of gyrodactylids, involving a combination of asexual, parthenogenetic and sexual production of multiple generations within the same individuals and hyperviviparity (Cable and Harris, 2002), protects embryos from Zn toxicity. However, our study was not designed to detect potential long term mutagenic or teratogenic effects of Zn.

When we examined the impact of Zn on parasite size, we found that the longer the period of exposure to Zn and the higher the concentration, the smaller the parasites were. This was observed in the mini-epidemics maintained at concentrations as low as 15 µg Zn/L for at least 5 days. More prolonged exposure to Zn results in smaller parasites that, in turn, give birth to even smaller offspring indicating a cumulative toxic effect. In interpreting data from this experiment, it is important to note the mixed age structure of the parasite populations in the mini-epidemics. Although we cannot exclude the possibility that the observed effects of Zn on morphometrics reflect a changing age-structure of the parasite population, *Gyrodactylus* spp. are not generally considered to "grow" in a traditional sense; rather, they are reported as full-size at birth but this has never been tested experimentally. Therefore, the Zn dependent effect on parasite size presumably reflects embryonic development of

the parasites; hence age-structure of the parasite population should not affect the average size of the parasite.

Taken together, the present results do not explain the parasite population dynamics observed in our first study (Gheorghiu *et al.*, 2006). We had reported improved parasite population growth at Zn concentrations up to 120 µg/L (Gheorghiu *et al.*, 2006) whereas our present results indicate reduced parasite survival and reduced reproduction over this range of Zn concentrations. Therefore, we reject the suggestion that waterborne Zn improves parasite growth and reproduction, and hypothesize that the host response against the parasite may be impaired as Zn concentration increases, especially after one week pre-exposure, thus providing a microenvironment permissive for parasite population growth. This hypothesis is currently under investigation. In conclusion, our data shows that elevated concentrations of Zn are toxic to *G. turnbulli*.

### 4.6 Acknowledgements

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**References**


Asch, H.L., Dresden, M.H., 1977. *Schistosoma mansoni*: effects of zinc on cercarial and schistosomule viability. J. Parasitol. 63, 80-86.

Bakke, T.A., Cable, J. and Harris, P.D., in press. The biology of gyrodactylid monogeneans: the "Russian- Doll Killers". Adv. Parasitol.

Bakke, T.A., Harris, P.D., Hansen, L.P. and Jansen, P.A., 1992. Host specificity and dispersal strategy in gyrodactylid monogeneans with particular reference to *Gyrodactylus salaris* (Plathyhelminthes, Monogenea). Dis. Aquat. Org. 13, 45-57.

Buchmann, K., Lindenstrom, T., 2002. Interactions between monogenean parasites and their fish hosts. Int. J. Parasitol. 32, 309-319.

Cable, J. and Harris, P.D., 2002. Gyrodactylid developmental biology: historical review, current status and future trends. Int. J. Parasitol. 32, 255-280.

Cable, J. and Tinsley, R.C., 1991. Intra-uterine larval development of the polystomatid monogeneans, *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis*. Parasitology 103, 253-266.

Cable, J., Scott, E.C.G., Tinsley, R.C. and Harris, P.D., 2002a. Behavior favoring transmission in the viviparous monogenean *Gyrodactylus turnbulli*. J. Parasitol. 88,183-184.

Cable, J., Tinsley, R.C. and Harris, P.D., 2002b. Survival, feeding and embryo development of *Gyrodactylus gasterostei* (Monogenea: Gyrodactylidae). Parasitology 124, 53-68.

Calabrese, E.J., Baldwin, L.A., 2003. Toxicology rethinks its central belief. Nature 421, 691- 692.

Canadian Council of Ministers of the Environment, 2005. Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life. http://www.ccme.ca/assets/pdf/wqg_aql_summary_table.pdf

Canadian Council on Animal Care, 2005. Guidelines on: the care and use of fish in research, teaching and testing. http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Fish/Fish%20Guidelines%20English.pdf

Dineley, K.E., Richards L.L., Votyakova, T.V., Reynolds, I.J., 2005. Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria. Mitochondrion 5, 55-65.

Eddy, F.B., Fraser, J.E., 1982. Sialic acid and mucus production in rainbow trout (*Salmo gairdneri* Richardson) in response to zinc and seawater. Comp. Biochem. Physiol. C. 73, 357-359.

Eisler, R., 1993. Zinc hazards to fish, wildlife and invertebrates: a synoptic review. U.S. Department of the Interior Fish and Wildlife Service. Patuxent Wildlife Research Center Biological Report 10. Contaminant Hazard Reviews Report 26. Laurel, Maryland 20708.

Evans, N.A., 1982a. Effect of copper and zinc upon the survival and infectivity of *Echinoparyphium recurvatum* cercariae. Parasitology 85, 295-303.

Evans, N.A., 1982b. Effect of copper and zinc on the life cycle of *Notocotylus attenuatus* (Digenea: Notocotylidae). Int. J. Parasitol. 12, 363-369.

Gheorghiu, C., Marcogliese, D.J. and Scott, M., 2006. Concentration-dependent effects of waterborne zinc on population dynamics of *Gyrodactylus turnbulli* (Monogenea) on isolated guppies (*Poecilia reticulata*). Parasitology 132, 225-232.

Handy, R.D., Eddy, F.B. and Romain, G., 1989. *In vitro* evidence for the ionoregulatory role of rainbow trout mucus in acid, acid/aluminum and zinc toxicity. J.Fish Biol. 35, 737-747.

Harris, P.D., Cable, J., Tinsley, R.C. and Lazarus, C.M., 1999. Combined ribosomal DNA and morphological analysis of individual gyrodactylid monogeneans. J. Parasitol. 85, 188-191.

Harris, P.D., Shinn, A.P., Cable, J. and Bakke, T.A., 2004. Nominal species of the genus *Gyrodactylus* v. Nordmann 1832 (Monogenea: Gyrodactylidae), with a list of principal host species. Syst. Parasitol. 59, 1-27.

Hogstrand, C. and Wood, C.M., 1996. The physiology and toxicology of zinc in fish. In: Taylor, E.W. (Ed.) Toxicology of aquatic pollution. Physiological, cellular and molecular approaches. Cambridge University Press, Cambridge, pp. 61-84.

Khan, R.A. and Kiceniuk, J.W., 1988. Effect of petroleum aromatic hydrocarbons on monogeneids parasitizing Atlantic cod, *Gadus morhua* L. Bull. Environ. Contam. Toxicol. 41, 94-100.

Khunyakari, R.P., Tare, V. and Sharma, R.N., 2001. Effects of some trace heavy metals on *Poecilia reticulata* (Peters). J. Environ. Biol. 22, 141-144.

Lester, R.J.G. and Adams, J.R., 1974. *Gyrodactylus alexandri*: reproduction, mortality, and effect on its host *Gasterosteus aculeatus*. Can. J. Zool. 52, 827-833.

McGeer, J.C., Szebedinszky, C., McDonald, D.G.and Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1. Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231-234.

Morley, N.J., Crane, M. and Lewis, J.W, 2001a. Toxicity of cadmium and zinc to miracidia of *Schistosoma mansoni*. Parasitology 122, 81-85.

Morley, N.J., Crane, M. and Lewis, J.W., 2001b. Toxicity of cadmium and zinc to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. Int. J. Parasitol. 31, 1211-1217.

Morley, N.J., Crane, M. and Lewis, J.W., 2002. Toxicity of cadmium and zinc mixtures to *Diplostomum spathaceum* (Trematoda: Diplostomidae) cercarial survival. Arch. Environ. Contam. Toxicol. 43, 28-33.

Morley, N.J., Crane, M. and Lewis, J.W., 2003. Effects of cadmium and zinc toxicity on orientation behaviour of *Echinoparyphium recurvatum* (Digenea: Echinostomatidae) cercariae. Dis. Aquat. Org. 56, 89-92.

Olstad, K., Cable, J., Robertsen, G. and Bakke, T.A., 2006. Unpredicted transmission strategy of *Gyrodactylus salaris* (Monogenea: Gyrodactylidae): survival and infectivity of parasites on dead hosts. *Parasitology 133, 33-41.*

Poléo, A.B.S., Schjolden, J., Hansen, H. and Bakke, T.A., Mo, T.A., Rosseland, B.O., Lydersen, E., 2004. The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). Parasitology 128, 169-177.

Rohlf, F.J. and Sokal, R.R., 1981. Statistical Tables, 2nd edition, 219 pp. W.H. Freeman and Company, NY.

Scott M.E., 1982. Reproductive potential of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). Parasitology 85, 217-236.

Scott M.E. and Anderson, R.M., 1984. The population dynamics of *Gyrodactylus bullatarudis* (Monogenea) within laboratory populations of the fish host *Poecilia reticulata*. Parasitology 89, 59-94.

Shephard, K.L., 1994. Functions for fish mucus. Rev. Fish Biol. Fish. 4, 401-429.

Smyth, J.D. and Halton, D.W., 1983. The Physiology of Trematodes. 2nd ed. Cambridge University Press, Cambridge.

Soleng, A., Poléo, A.B.S., Alstand, N.E.W. and Bakke, T.A., 1999. Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. Parasitology 119, 19-25.

Vallee, B.L. and Falchuk, K.H., 1993. The biochemical basis of zinc physiology. Physiol. Rev. 73, 79-118.

## CONNECTING STATEMENT BETWEEN CHAPTER 4 AND CHAPTER 5

One major result of our first study on *Gyrodactylus turnbulli* on isolated guppies exposed to waterborne Zn (0 to 240 µg/L) was that parasite population growth was improved at Zn concentrations up to 120 µg/L (Gheorghiu *et al.*, 2006). These results suggested that concentrations up to 120 µg Zn /L are beneficial for the survival and/or reproduction of the parasite. However, this hypothesis was not supported by our follow-up study in which we recorded direct toxicity of Zn on both parasite survival and morphometrics at concentrations of 30 and 120 µg Zn/L (Gheorghiu *et al.*, in press). Our alternative hypothesis was that waterborne Zn impairs the local host defense mechanisms against the gyrodactylid infection.

The next manuscript describes a set of experiments designed to investigate temporal histological changes in fish epidermis induced by: (1) a range of sublethal concentrations of waterborne Zn exposure alone; (2) the gyrodactylid infection alone; (3) combined waterborne Zn exposure and infection with *Gyrodactylus*.

# CHAPTER 5

# TEMPORAL DYNAMICS OF EPIDERMAL RESPONSES OF GUPPIES (*POECILIA RETICULATA*) TO TWO EXTERNAL STRESSORS: WATERBORNE ZINC AND *GYRODACTYLUS TURNBULLI* (MONOGENEA) INFECTION[i]

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Running Title: Epidermal responses of guppies to zinc and/or *Gyrodactylus*

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### 5.1 Abstract

This study assessed the histological changes in the epidermis of guppies induced by waterborne zinc (Zn) and an ectoparasite (*Gyrodactylus turnbulli*). Infected and uninfected guppies were exposed to 0, 15, 30, 60 or 120 µg Zn/L and monitored over three to four weeks. Our results demonstrate that the fish epidermis responded immediately to *G. turnbulli* infection with a rapid increase in epidermal thickness and an increase then decline in mucous cell numbers, after which the the epidermis remained thick and mucous numbers remained low. Waterborne Zn induced rapid release of mucous and a decrease in mucous cell numbers; thereafter mucous release fluctuated at a rate that varied with Zn concentration. Epidermal thickness began to fluctuate only after 6 days Zn exposure. The mucin composition also responded rapidly to Zn, shifting from a mixture of acidic and neutral mucins at 0 µg/L to acidic at all concentrations except 30 µg/L but was unaffected by *G. turnbulli* alone. When Zn and infection were combined, Zn exposure dominated the initial response as shown by immediate decrease in mucous cell numbers, whereas the infection dominated the subsequent changes as evidenced by thickening of the epidermis and maintenance of normal mucin composition.



*Keywords:* Waterborne zinc; Guppy; Gyrodactylids; Epidermal histology; Mucous cells; Mucins.

## 5.2 Introduction

*Gyrodactylus* spp. are important monogenean ectopathogens in aquaculture, fisheries and hobbyists markets, affecting many families of marine and freshwater teleost fish and causing mortality both directly and indirectly through secondary bacterial or fungal infections (Kearn, 1998; Cone, 1999). These small epidermal browsers cause mechanical disruption of the epithelium while feeding and moving over the skin and fins (Kearn, 1998; Cone, 1999). They are viviparous and transmission occurs by direct fish-to-fish contact (Cable, Harris & Tinsley, 1996; Bakke, Hansen & Jansen, 1992). Shortly after arrival of a gyrodacytlid on the fish, the parasite gives birth (the lag phase of the infection). As reproduction continues, the parasite population increases exponentially (exponential growth phase). On some fish, the parasite numbers continue to increase until the damage induced by infection kills the fish. On others, the parasite elicits a host response (Buchmann and Bresciani, 1998, 1999; Buchmann, 1999; Harris, Soleng & Bakke, 1998; Buchman and Lindenstrøm, 2002) which impairs parasite survival and reproduction. Parasite numbers decline (recovery period) and the fish is able to cure the infection. The death or recovery of the infected fish, as well as the duration of each phase of infection, are highly variable depending on the intensity of the host response to parasite assault (Scott and Robinson, 1984; Scott, 1985; Richards and Chubb, 1996, 1998) and environmental factors such as water temperature (Scott and Nokes, 1984) and pollutants (Khan and Kiceniuk, 1988; Soleng, Poléo, Alstand, & Bakke, 1999; Poléo, Schjolden, Hansen, Bakke, Mo, Rosseland & Lydersen,2004; Gheorghiu, Marcogliese & Scott, 2006).

Among the few studies on the impact of heavy metals on *Gyroactylus*, the results have varied depending on the host and parasite species. Soleng *et al.* (1999) and Poléo *et al.* (2004) recorded a negative impact on *G. salaris* populations on Atlantic salmon exposed to either waterborne aluminium (50 to 200 µg Al/L) or zinc (50 - 400 µg Zn/L), whereas the host appeared to be unaffected. Our previous study (Gheorghiu *et al.*, 2006) on *G. turnbulli* on isolated guppies exposed to waterborne Zn (0 to 240 µg/L) showed improved parasite population growth at Zn concentrations up to 120 µg/L. These results suggested that elevated Zn directly benefited survival and/or reproduction of the parasite, but this was not supported by our follow-up study in which we recorded direct toxicity of Zn on both parasite survival and morphometrics at concentrations of 30 and 120 µg Zn/L (Gheorghiu, Cable, Marcogliese & Scott, 2007). An alternative explanation for the more rapid parasite population growth with increasing Zn concentrations is that waterborne Zn impairs the local host defense mechanisms against the gyrodactylid infection. Fish respond to *Gyrodactylus* with increased mucus secretion (Lester and Adams, 1974; Scott and Anderson, 1984) and thickening of the epithelium through an increase in the number of cell layers (Appleby, Mo & Aase, 1997) and hyperplasia of epithelial cells (Wells and Cone, 1990). As infection progresses, mucous cell density may decrease (Wells and Cone, 1990; Sterud, Harris & Bakke, 1998) or increase (Barker, Cone & Burt, 2002). In addition, fish respond to Zn with increased mucous release containing acidic mucins that bind and precipitate Zn, thus regulating its absorption by preventing it from reaching the uptake surfaces (Handy, Eddy & Romain, 1989; Shephard, 1994).

Although *in vitro* studies indicate that the key host anti-gyrodactylid factor thought to be responsible for clearing the infection is the complement system (Buchman and Bresciani, 1998, and Harris *et al.*, 1998) *in vivo* survival and reproduction of *Gyrodactylus* depend on the proper function of the epidermal cells that influence the amount and composition of mucous and the content of both parasite chemoatractants and host anti-parasitic factors (Buchman, 1999; Buchman and Lindenstrøm, 2002). The fish epidermis consists of epithelial and secretory cells, and is covered by a layer containing mucus secreted by mucous (goblet) cells as well as the sloughed superficial layer of epidermal cells (Whitear, 1986). Both epithelial and secretory cells differentiate from a multipotent progenitor cell, and as new cells appear, the previously differentiated ones are gradually pushed towards the outer layer of the epidermis. The precursor mucous cell contains granules of neutral mucins exclusively (Sinha and Chakravorty, 1982). Under normal conditions, some of the neutral mucopolysaccharide granules then switch into acidic mucopolysaccharide granules and new acid mucopolysaccharides are synthesized within the cell. In mature mucous cells, these acidic and neutral polysaccharide granules fuse forming a complex mixture of both acidic and neutral mucins (Sinha and Chakravorty, 1982). There are spatial and temporal differences in epithelium thickness, in the density of mucous cells on the surface of a fish, and in the production and composition of mucus, depending on the species, age, state of health, nutritional status, and quality of the environment (Roberts and Bullock, 1980). Hence, the epidermis is a metabolically active tissue that quickly forms stable physical and/or chemical barriers against invading microorganisms and/or soluble contaminants (Iger,

Jenner & Wendelaar Bonga, 1994; Buchman and Lindenstrøm, 2002). Thus, we hypothesize that the dynamics of *G. turnbulli* on fish simultaneously exposed to waterborne Zn may be related to epidermal responses through the mucous production.

In the present study, we recorded the temporal histological changes in fish epidermis induced by: (1) a range of sublethal concentrations of waterborne Zn exposure alone; (2) the gyrodactylid infection alone; (3) combined waterborne Zn exposure and infection with *Gyrodactylus* using guppies and the host-specific *G. turnbulli* as our lab model. Guppies (*Poecilia reticulata*) are useful test animals in aquatic experiments because they are easy to maintain and breed under laboratory conditions and they are able to survive at very high concentrations of Zn (Widianarko, van Gestel, Verweij, & van Straalen, 2000; Widianarko, Kuntoro, van Gestel, Verweij & van Straalen, 2001). Also *G. turnbulli* burdens can be repeatedly monitored over time on individual hosts, as the parasite lives on the skin and fins.

## 5.3 Material and methods

### *5.3.1 General methods*

The experiments were done on guppy fry of 0.5-1.0 cm standard body length, bred in our laboratory from a strain of feeder guppies purchased from a local pet store, and naïve to *Gyrodactylus*. The experimental fry were maintained in individual rectangular plastic containers in 200 ml waterborne Zn solution at 25°C with 16 h light:8 h dark cycle and were fed on a Nutrafin Max Complete Flake diet once a day.

Sublethal concentrations for experimental waterborne Zn solutions were selected relative to 30 µg/L Zn, the maximal admissible limit for aquatic life according to Canadian Council of Ministers of the Environment (2005). They were prepared according to the method described by Gheorghiu *et al.* (2006) by adding 0 (as control), or 15, 30,, 60 or 120 µg/L Zn to artificial freshwater that contained 8 µg Zn /L, the concentration of waterborne Zn in the guppy breeding tanks. To maintain relatively constant Zn concentrations throughout the experiments, Zn solutions were replaced every two days (Gheorghiu *et al.*, 2006).

The strain of *G. turnbulli* was initially isolated from infected guppies from a local supplier, identified according to Harris, Cable, Tinsley & Lazarus (1999) and maintained by weekly addition of naïve fish into infected stock populations. The experimental fry were infected with 3 parasites transferred on a scale or piece of fin from an infected donor onto the caudal peduncle of a naïve recipient, as previously described (Scott, 1982; Gheorghiu *et al.*, 2006), and then monitored daily using a Nikon stereomicroscope with cold light while anaesthetized for a maximum of 5 min in 50 ml of 0.02% tricaine methanesulfonate (Finquel MS222, Argent Chemical Laboratories, Washington) buffered to a neutral pH with $NaHCO_2$.

All procedures were approved by a McGill University Animal Care Committee, in accordance with guidelines of the Canadian Council on Animal Care (2005).

*5.3.2 Experiment 1: Dynamics of epidermal responses to Zn exposure*

Experimental fish were randomly assigned to one of the five waterborne Zn solutions. Mucous production was monitored on fish exposed to 15-120 µg Zn /L (n=27 fry/concentration), sampled on days 0, 3, 6, 9, 12, 15, 18, 21 and 28 post-exposure. At each time point, a different set of 3 fish / concentration was anaesthetized and mucous release was recorded under the stereomicroscope using a visual score from 1 (minimal) to 5 (maximal). The temporal changes induced by Zn alone were recorded on a separate set of fry exposed to 0-120 µg Zn /L. Three fry / concentration / day were sampled at the same time points as above. The fry were killed in 0.03% MS22, fixed for 24 h in Bouin's solution, washed two times and then preserved in 70% ethanol. Paraffin cross-sections (5 µm) were cut and stained using either hematoxylin and eosin (HE) for overall structure, or combined periodic acid-Schiff with Alcian Blue 2% (PAB) to differentiate between neutral polysaccharides and acidic mucopolysaccharides contained in the mucous cell (Tibbetts, 1997). When stained with PAB, the large swollen mature mucous cells were (1) purple if they contained both neutral and acidic mucins, (2) blue if they contained acidic mucins, or (3) magenta if they contained neutral mucins (Bancroft and Stevens, 1982). The sections were examined with a Nikon compound microscope at 425x magnification. We recorded epidermal thickness, number of epidermal cell layers, and number of mature mucous cells, their size, location, and mucin composition from 4 lateral fields of view of 0.24 $mm^2$ each, randomly chosen in each of three serial sections of the caudal peduncle per fish.

*5.3.3 Experiment 2: Dynamics of epidermal responses to gyrodactylid infection alone or in combination with Zn exposure*

To test the effect of infection alone, guppies were experimentally infected with 3 parasites and isolated in plastic containers with 200 ml artificial freshwater. The parasite population was then monitored daily on each fish. The epidermal response to infection was recorded in three experimental fish / concentration, at each of the following phases of the infection: pre-infection; the lag period (less than 6 parasites per fish); early exponential growth (approximately 20 parasites per fish); mid exponential growth (approximately 50 parasites per fish; and late exponential growth (approximately 100 parasites per fish). The fish were processed for histological analysis as described for Experiment 1.

The combined effects of infection and Zn exposure were tested in guppies experimentally infected with three *G. turnbulli* each and then immediately placed in one of the five Zn solutions chosen at random. Parasites were counted daily and three fish / concentration / phase of infection were prepared for histological assessment at each of the phases of infection as described above.

*5.3.4 Statistical analysis*

The temporal effect of waterborne Zn alone or together with *Gyrodactylus* infection on the guppy epidermis was assessed using $\chi^2$ for categorical variables (mucus production scores, mucin composition, mucous cell location) and two-way ANOVA using PROC GLM for parasite burdens. Continuous histological variables were analyzed by two-way ANOVA using PROC MIXED with a nested model that controlled for degrees of freedom, and post-hoc contrasts across time

/ phase of infection within each Zn concentration using LSMEANS with the SLICE option. Linear regression analysis was used to examine the relationship between epidermal thickness and number of cell layers. The mean and SE are reported. Analyses were performed using SAS Version 9.1 software. The level of significance was established at $p<0.05$; statistics are reported only for significant effects.

## 5.4 Results

### *5.4.1 Experiment 1: Dynamics of epidermal responses to Zn exposure*

All histological parameters were significantly affected by waterborne Zn, by duration of Zn exposure and by the interaction between the two factors, whereas for uninfected fish kept in 0 µg Zn/L, none of the histological parameters differed over time. The epidermis of guppy fry kept in 0 µg Zn/L had 2-3 layers of epidermal cells, a thickness of 8.4 ± 0.23 µm and 3.15 ± 0.4 mature mucous cells per 0.24 $mm^2$, most of which were located on the external layer, and which contained a mixture of neutral and acidic mucins (Fig. 5.1A).

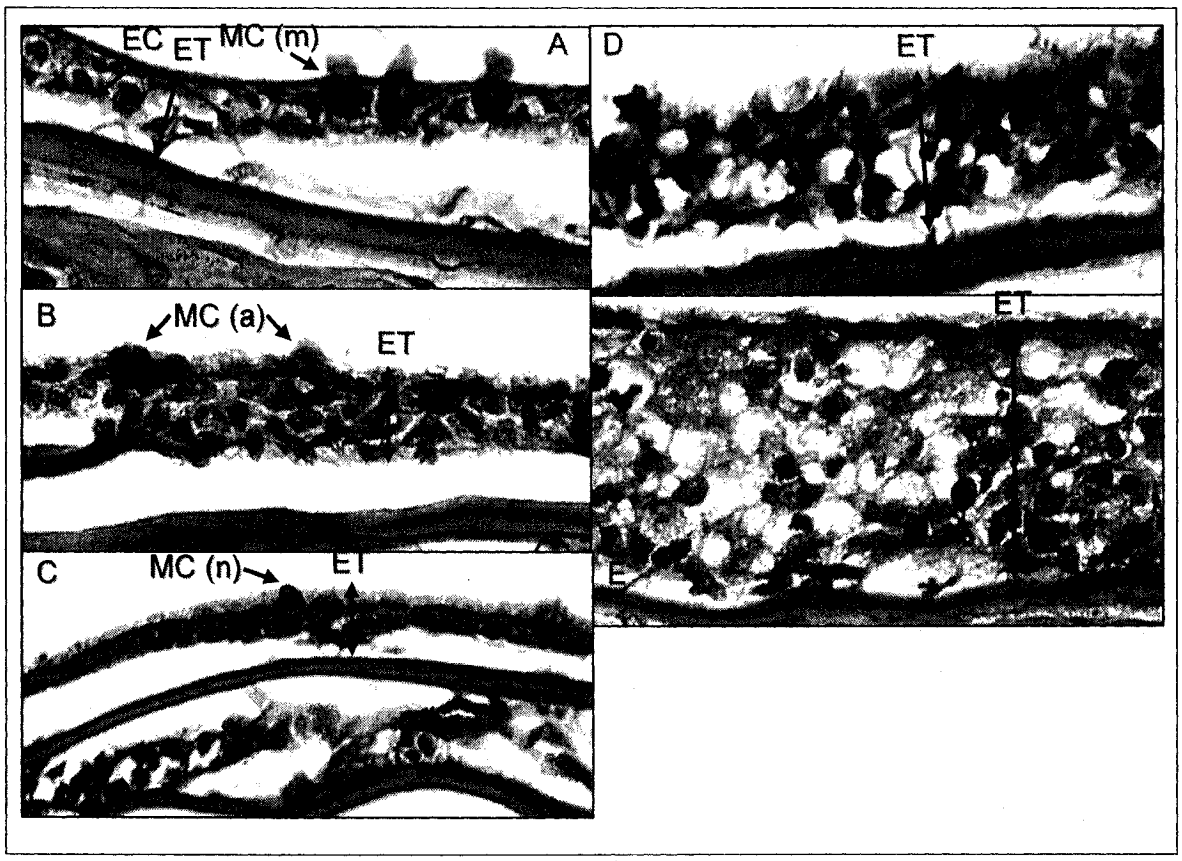
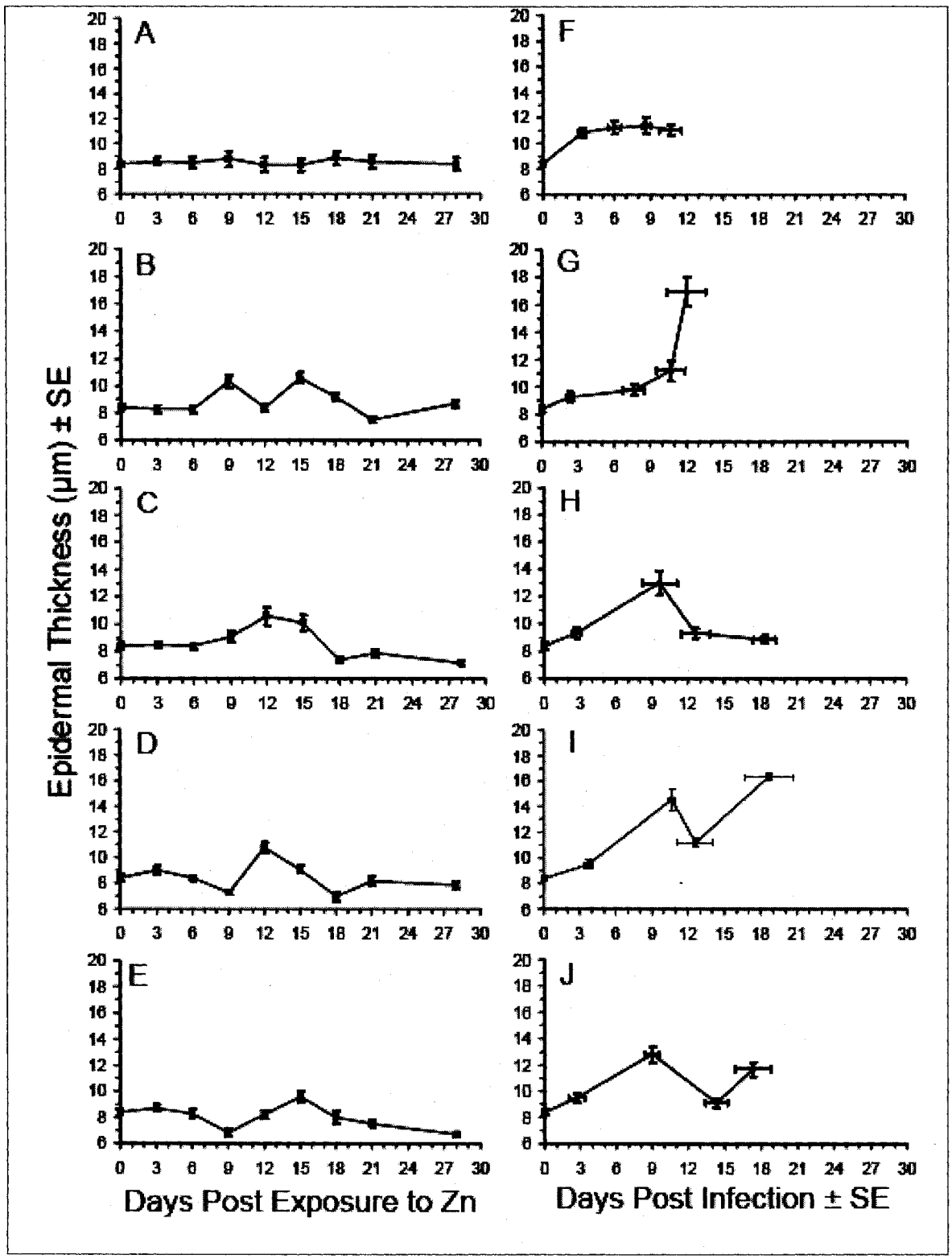



Fig. 5.1 Histological images of epidermis of guppies (PAB staining, 425x): (A) Epidermis of control fish, uninfected and unexposed to Zn. ET – thickness of epidermis, EC - epithelial cells, MC (m) - mucous cells with a mixture of neutral and acidic mucins, S - scale. (B) Mucous cells contained only acidic mucins – MC (a) in fish exposed to 15 µg Zn/L. (C) Mucous cells contained only neutral mucins, MC (n), in fish exposed to 30 µg Zn/L. (D) Moderate thickening of the epidermis in fish infected with *Gyrodactylus*. (E) Extreme thickening of the epidermis in fish exposed to 15 µg Zn/L and infected with 100 *Gyrodactylus*.

5.4.1.1 Epidermal thickness and cell layers

Exposure to waterborne Zn did not influence epidermal thickness over the first 6 days regardless of Zn concentration. Low concentrations of Zn (15 and 30 µg Zn/L) then induced an increase in epidermal thickness (Fig. 5.2B) followed by a decline (Fig. 5.2B, C). In contrast, at higher concentrations (60 and 120 µg Zn/L), the epidermis became thinner on day 9 post exposure before increasing and then declining again (Fig. 5.2D, E). Furthermore, in fish kept in higher concentrations than 30 µg Zn/L, on day 28 post exposure, the thickness was decreased compared with the initial value. The number of epithelial cell layers followed similar patterns as the epidermal thickness (Appendix C). In addition, regression analysis revealed a positive relationship between epidermal thickness and the number of epidermal cell layers ($p<0.0001$). These patterns demonstrate that Zn exposure induced fluctuations in the thickness of the epidermis and the number of cell layers over time. The rate of fluctuation differed as concentration of Zn increased such that the initial peak of increase in thickness was delayed with increasing concentration of Zn and repeated fluctuations were only recorded at 15 µg Zn/L.

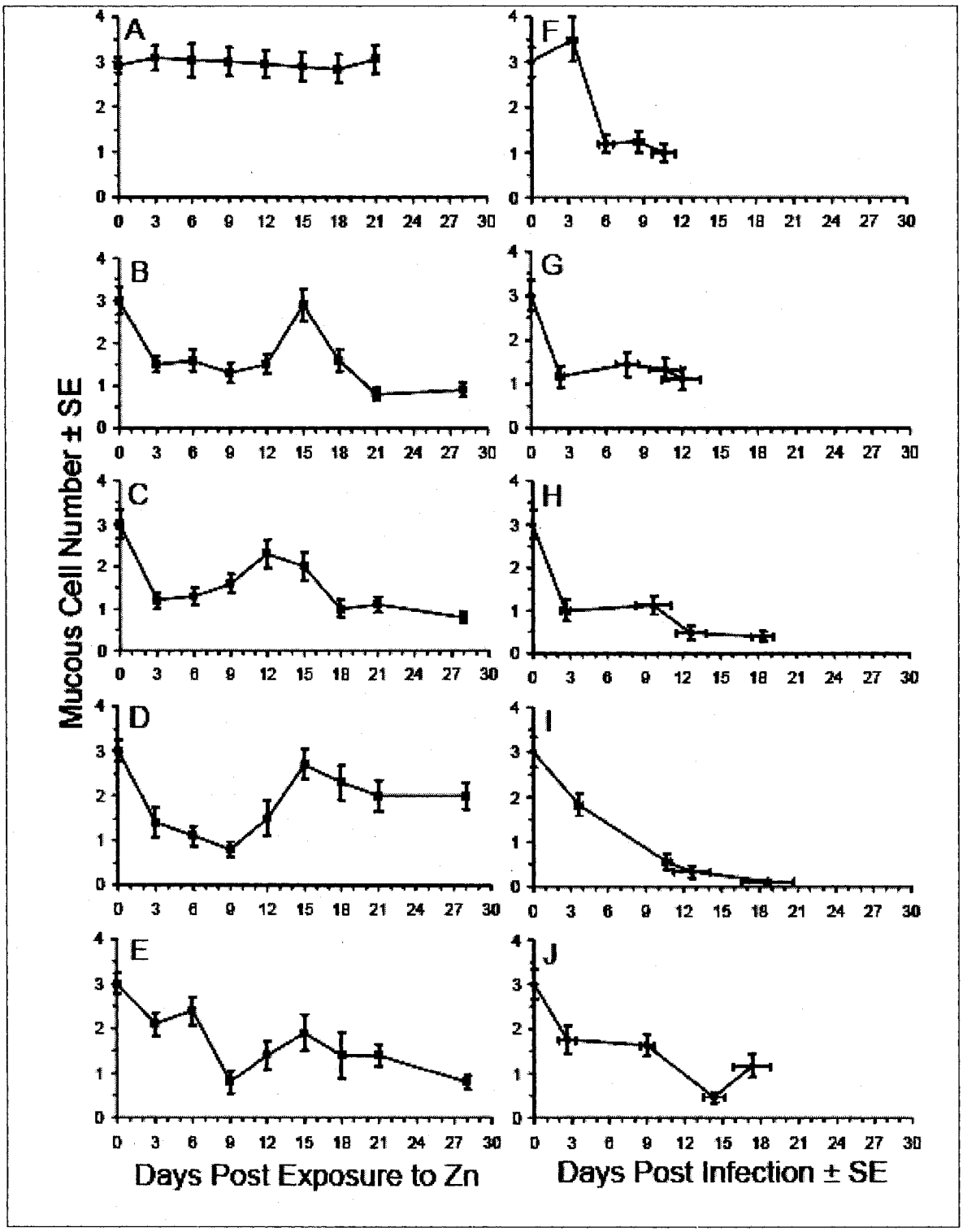
Fig. 5.2 Temporal dynamics of epidermal thickness of guppies in response to Zn and/or *Gyrodactylus*. (A) 0 µg Zn/L; (B) 15 µg Zn/L; (C) 30 µg/L; (D) 60 µg Zn /L; (E) 120 µg Zn/L; (F) *Gyrodactylus*; (G) *Gyrodactylus* and 15 µg Zn/L; (H) *Gyrodactylus* and 30 µg Zn/L; (I) *Gyrodactylus* and 60 µg Zn/L; (J) *Gyrodactylus* and 120 µg Zn/L. Main effects: Zn, $p<0.0001$; time, $p<0.0001$; Zn*time, $p<0.0001$.


A
B
C
D
E
F
G
H
I
J
Epidermal Thickness (µm) ± SE
Days Post Exposure to Zn
Days Post Infection ± SE

5.4.1.2 Mucous response

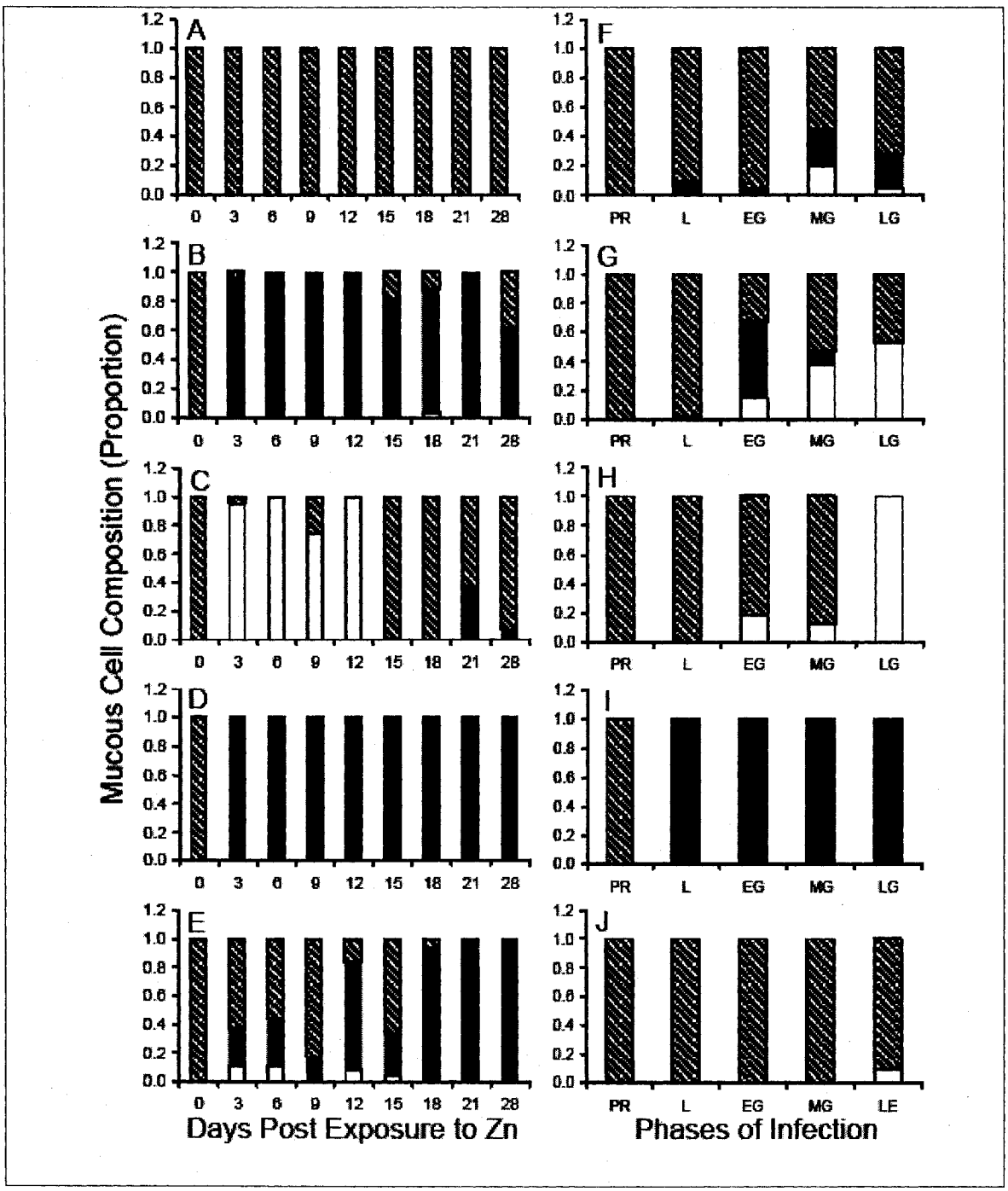
Compared to control fish exposed to 0 µg Zn/L, Zn induced a rapid 25-50% decline in the number of mucous cells within 3 days (Fig. 5.3B-E), regardless of Zn concentration. The numbers were gradually restored (except at 120 µg Zn/L) over the next 9-12 days but then declined again. Similarly mucous cell size declined and then gradually returned to normal (Appendix D). Cells in fish exposed to 15 µg Zn/L subsequently became smaller (day 3 and 18) and returned to normal size around day 15 and 28, respectively. At concentrations higher than 30 µg Zn/L, the cells remained half the size of cells in unexposed fish, even after 28 days (Appendix D). All mature mucous cells were located in the outer layer of the epidermis, regardless of Zn concentration or duration of exposure (Fig. 5.1B, C). The only exception was for guppies exposed to 120 µg Zn/L where on average, 19.1 % ± 2.4 of mucous cells were localized in the inner layers.

Fig. 5.3 Temporal dynamics of mucous cell number in response to Zn and/or *Gyrodactylus*. (A) 0 µg Zn/L; (B) 15 µg Zn/L; (C) 30 µg/L; (D) 60 µg Zn /L; (E) 120 µg Zn/L; (F) *Gyrodactylus*; (G) *Gyrodactylus* and 15 µg Zn/L; (H) *Gyrodactylus* and 30 µg Zn/L; (I) *Gyrodactylus* and 60 µg Zn/L; (J) *Gyrodactylus* and 120 µg Zn/L. Main effects: Zn, $p<0.0001$, time, $p<0.0001$, Zn*time, $p<0.0001$.


A
B
C
D
E
F
G
H
I
J
Mucous Cell Number ± SE
Days Post Exposure to Zn
Days Post Infection ± SE

In control fish not exposed to Zn, mature mucous cells contained a mixture of acidic and neutral mucins (Fig. 5.1A; Fig. 5.4A). Exposure to 15 µg Zn/L for only 3 days induced a loss of neutral mucins and a complete shift to acidic mucins that remained until day 28 (Fig. 5.1B, Fig. 5.4B). The same pattern was seen in fish exposed to 60 µg Zn/L (Fig. 5.4D). In contrast, at 30 µg Zn/L, mucin composition shifted to neutral within 3 days (Fig. 5.1E) and then returned to control mixed composition on day 15 (Fig. 5.4C), whereas fish exposed to 120 µg Zn/L demonstrated a very gradual shift from mixed mucins to acidic over a period of 18 days (Fig. 5.4E).

Fig. 5.4 Frequency distribution of mucous cell composition in response to Zn and/or *Gyrodactylus*. (A) 0 µg Zn/L; (B) 15 µg Zn/L; (C) 30 µg/L; (D) 60 µg Zn /L; (E) 120 µg Zn/L; (F) *Gyrodactylus*; (G) *Gyrodactylus* and 15 µg Zn/L; (H) *Gyrodactylus* and 30 µg Zn/L; (I) *Gyrodactylus* and 60 µg Zn/L; (J) *Gyrodactylus* and 120 µg Zn/L.. $p<0.0001$. Striped bars – proportion of mucous cells with mixture of neutral and acidic mucins; solid bars – proportion of mucous cells with acidic mucins; clear bars - proportion of mucous cells containing neutral mucins. PR- pre-infection and pre-exposure to Zn; L - the lag period (less than 6 parasites per fish); EG -early exponential growth (approximately 20 parasites per fish); MG - mid exponential growth (approximately 50 parasites per fish); and LG - late exponential growth (approximately 100 parasites per fish).




Among fish exposed to 15 – 120 µg Zn/L, mucous production was significantly affected by Zn concentration, by duration of exposure and by the interaction between these two factors. Our semi-quantitative scores on mucous

production revealed that mucous production increased then declined repeatedly over the first 18 days of Zn exposure, and then remained low until day 28 (Fig. 5.5A-D). The highest scores were recorded in fish kept in 15 and 30 µg Zn/L (Fig. 5.5A, B), which showed almost similar patterns. Intriguingly, mucous release was dampened at 60 µg Zn/L, between days 6 and 12 (Fig. 5.5C). Also, at 120 µg Zn/L mucous discharge remained elevated for the first 9 days, then declined and increased again before remaining low between day 18 and day 28 (Fig. 5.5D).

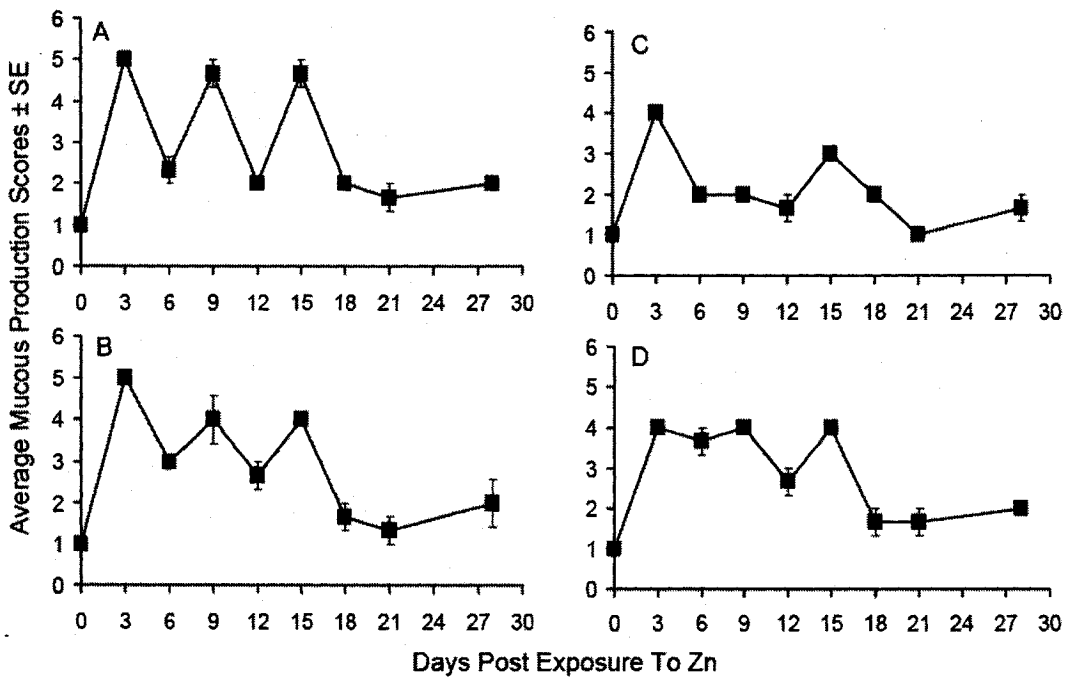



Fig. 5.5 Average mucous production scores in response to (A) 15 µg Zn/L, (B) 30 µg Zn/L, (C) 60 µg Zn/L or (D) 120 µg Zn/L. Scores range from 1 (low) to 5 (high). Main effects: Zn, $p<0.0001$; time, $p<0.0001$; Zn*time, $p=0.0002$.

*5.4.2 Experiment 2: Dynamics of epidermal responses to gyrodactylid infection alone or in combination with Zn exposure.*

5.4.2.1 Epidermal thickness and cell layers

Infection alone induced thickening of the epidermis within 3 days which was then maintained at the same level throughout exponential growth of the parasite population (Fig. 5.1D, 5.2F). This rapid response did not occur in infected fish concurrently exposed to Zn (Fig. 5.2G-J). Instead, the combined stress of infection and Zn induced a 50% increase in epidermal thickness only after 10 days (Fig. 5.2G-J) despite the fact that parasite numbers had only increased to 20. Epidermal thickness subsequently declined to baseline levels as exponential growth of the parasite population continued in fish exposed to 30 µg Zn/L (Fig. 5.2H). At higher Zn concentrations, as the number of parasites continued to increase, the thickness declined and then increased again (Fig. 5.2I-J). In marked contrast, the epidermis continued to thicken in those fish exposed only to 15 µg Zn/L, reaching twice the value of uninfected, unexposed fish (Fig. 5.1E, 5.2G). The number of cell layers mirrored this pattern indicating hyperplasia of epithelial cells (Appendix C).

5.4.2.2 Mucous response.

As in uninfected fish, the majority of mature mucous cells were present on the external surface of the epidermis, although as infection progressed some mature cells were detected within the epidermis. The exception was for fish exposed to 15 µg Zn/L, where the proportion of mucous cells localized in the

inner layers of the epidermis mirrored the change in parasite numbers, gradually increasing as the parasite population increased to 100, where 92% of the cells were located internally (Table 5.1).

Table 5.1 Percentage of mature mucous cells located on the external surface of the epidermis of guppies exposed concurrently to *Gyrodactylus turnbulli* infection and waterborne Zn ± SE.

| Zn concentration | Phases of Infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pre-exposure | Lag | Early Exponential Growth | Mid Exponential Growth | Late Exponential Growth | Prolonged Lag | Mid Recovery |
| 0 µg Zn/L | 98 ± 1 | 97 ± 2 | 63 ± 8 | 73 ± 7 | 78 ± 7 | NA | 91 ± 5 |
| 15 µg Zn/L | 98 ± 1 | 100 ± 0 | 85 ± 5 | 40 ± 7 | 8 ± 4 | NA | 68 ± 7 |
| 30 µg Zn/L | 98 ± 1 | 97 ± 3 | 97 ± 3 | 76 ± 11 | 86 ± 10 | 77 ± 6 | 67 ± 33 |
| 60 µg Zn/L | 98 ± 1 | 95 ± 3 | 64 ± 10 | 100 ± 0 | 80 ± 20 | 93 ± 3 | 100 ± 0 |
| 120 µg Zn/L | 98 ± 1 | 100 ± 0 | 97 ± 2 | 100 ± 0 | 83 ± 6 | 99 ± 1 | 66 ± 9 |

Exposure of infected fish to Zn induced a rapid and dramatic drop in the number of mucous cells within only 3 days (Fig. 5.3G-J) whereas infection alone did not exert such an immediate effect (Fig. 5.3F). However, as with infection alone, mucous cell numbers remained depressed throughout the period of infection after day 3 (Fig. 5.3F-J). Notably, virtually no mucous cells were observed when parasite numbers reached 100 at 60 µg Zn/L (Fig. 5.3I, day 19). In general, mucous cell size decreased in infected fish exposed to Zn, a pattern similar to changes in total numbers of mucous cells. However, in fish maintained at 60 µg Zn/L, cell size gradually increased to normal during the exponential growth phase of infection (Appendix D).

Infection alone exerted a modest effect on the composition of mucins only during the mid-exponential growth phase when approximately 25% of cells contained only acidic mucins, 25% contained only neutral mucins (Fig. 5.4F). An earlier and more marked pattern in shift to more acidic (50% during early exponential growth phase) or neutral (50% during late exponential growth phase) mucins emerged in infected fish exposed to 15 µg Zn/L (Fig. 5.4G). At concentrations of 30 and 120 µg Zn/L (Fig. 5.4H, J), mucin composition appeared similar to control uninfected fish not exposed to Zn, except for fish exposed to 30 µg Zn/L when parasite numbers were maximal and 100% of mucous cells contained neutral mucins (Fig. 5.4H). Perhaps the most intriguing observation was that mucin composition in infected fish exposed to 60 µg Zn/L was consistently acidic (Fig. 5.4I), as was observed for uninfected fish exposed to the same concentration of Zn.

## 5.5 Discussion

Our results showed that fish epidermis responded differently depending on the stressor and on the duration of exposure and/or phase of infection. Zn alone induced a rapid response that was not sustained for the duration of the exposure. Rather it fluctuated until day 18, and then remained constant. *Gyrodactylus* infection induced an immediate moderate thickening of the epidermis, and a reduction in mucous cells as the parasite numbers reached 20 and beyond. When combined, Zn exposure dominated the initial response, whereas the infection dominated the subsequent changes. In discussing the host response to Zn, to infection and to the combined stresses, we divide the temporal pattern into three periods: the immediate (within the first 3 days), the intermediate (4-18 days) and the chronic response (beyond 18 days).

Immediately following Zn exposure, we observed a rapid elevation in mucous scores reflecting the release of mucus onto the fish surface (Fig. 5.5). This release of mucus would protect fish from Zn toxicity by trapping excessive Zn and thus preventing its entry into underlying tissues (Shephard, 1994). We also observed a concurrent decrease in mucous cell numbers (Fig. 5.3B-E), consistent with that observed by other researchers in response to a wide range of pollutants (Iger, Abraham, Dotan, Fattal & Rahamim, 1988; Iger *et al.*, 1994; Benedetti, Albano & Mola, 1989; Iger and Wendelaar Bonga, 1993). The chemical composition of mucins changed dramatically with Zn concentration. Whereas mucous cells in fish unexposed to Zn contained a mixture of neutral and acidic mucins (Fig. 5.4A), mucous cells in fish exposed to 15 and 60 µg Zn/L contained only acidic mucins (Fig. 5.4B, D). Acidic mucins are reportedly

protective against Zn (Handy *et al.*, 1989; Shephard, 1994) and the Golgi cisternae in mucous cells switch to producing strongly acidic mucins in response to a variety of stresses (Triebskorn, Christensen & Heim, 1998). Interestingly, mucous cells exposed to 30 µg Zn/L contained only neutral mucins (Fig. 5.4C). In response to 120 µg Zn/L, mucin composition 3 days after exposure to Zn was very similar to that of unexposed fish (Fig. 5.4E). Perhaps a shift in composition in these fish occurred earlier than day 3 or perhaps there was a more marked upregulation of differentiation of new mucous cells, suggested by the presence of internally localized mucous cells. Thus, although at day 3, mucus was released at 30 and 120 µg Zn/L its chemical composition may not have been as protective as at other concentrations of Zn. Maximal initial protective response of mucous against Zn presumably occurred at 15 and 60 µg Zn/L.

An immediate response of fish to *G. turnbulli* infection also occurred but characteristics differed compared with the response to Zn. *Gyrodactylus* initially induced a modest increase in mucous cell numbers with no shift in mucin composition, in contrast to the rapid decrease and change in composition in response to most Zn concentrations. In addition, infection induced a rapid thickening of the epidermis even though parasite numbers at this time were very low.

The initial response to combined Zn and infection exposure appears to have been driven mainly by Zn, especially at concentrations higher than 15 µg Zn/L. Mucous cell numbers decreased, mucin composition shifted to acidic (though only at 60 µg Zn/L), but epidermal thickness was unaffected, all features of the immediate host response to Zn. Given the low numbers of parasites on the

fish at this early stage of infection, it was not surprising that Zn emerged as the more dominant driver of the host response. However, despite the low parasite intensity, our data indicate that infection reversed the shift in mucin composition in response to low concentrations of Zn (15 and 30 µg Zn/L). To our knowledge, these initial skin responses to gyrodactylids and to the combined effect of gyrodactylids and Zn have not been previously documented.

After the immediate response to Zn alone, mucous release began to fluctuate as did epidermal thickness and mucous cell numbers. At 15 µg Zn/L, mucous release and epidermal thickness showed a repeated pattern of fluctuation (Fig. 5.5A, 5.2B), as did mucous release at 30 µg Zn/L (Fig. 5.5B). At higher concentrations, all three parameters fluctuated over time, but did not show evidence of repeated cycles (Fig. 5.2D-E, Fig. 5.3D-E, Fig. 5.5C-D). The fluctuations could be due to the time delay between differentiation of multipotent progenitor cells induced by the sloughing of epidermal cells, and the migration of the new cells to the surface of the epidermis. Similar fluctuations in epidermal thickness have been recorded in rainbow trout exposed to Rhine waters containing a mixture of pollutants including 30 µg Zn/L (Iger *et al.*, 1994). Between days 6 and 18, fish exposed to 15 and 60 µg Zn/L continued to produce acidic mucins as they had at day 3 (Fig. 5.4B, D). Surprisingly, however, at 120 µg Zn/L the timing of the shift to acidic mucins in these cells was delayed compared to the other concentrations (Fig. 5.4E). This was not the result of a generalized lack of response of mucous cells, because mucous production scores remained elevated over the first 9 days of Zn exposure (Fig. 5.5D). Sustained synthesis and discharge is an energy-demanding process (Shephard,

1994) and conversion of neutral to acidic mucins as well as synthesis of more acidic mucins may also be energetically costly. When demands for mucous production are high (as at 120 ug Zn/L), an energetic trade-off may occur between up-regulating mucous production and up-regulating the synthesis of acidic mucins. This hypothesis is consistent with the elevated mucous production and replacement of depleted mucous cells but the absence of a shift to acidic mucins that we observed in fish exposed to 120 ug Zn/L.

After the initial phase of parasite establishment, infection alone induced a significant reduction in mucous cell numbers and both thickening of the epidermis and low numbers of mucous cells were maintained at the same level for the whole exponential growth phase with no evidence of recurring fluctuations. Similar epidermal responses have been reported in response to a variety of species of *Gyrodactylus*, regardless of the *Gyrodactylus* – host system taken in study, the type of study (field or experimental), or the intensity of infection at the moment of sampling (Wells and Cone, 1990; Appleby *et al.*, 1997; Sterund *et al.*, 1998). The skin damage induced by these epithelial browsers destroys both epithelial and mucous cells in the epidermis (Wells and Cone, 1990; Sterud *et al.*, 1998). In response, multipotent progenitor cells are more likely to differentiate into epithelial cells at the expense of mucous cells, simply because epithelial cells are the predominant cell type. Hyperplasia of epithelial cells will in turn cause thickening of the epidermis despite the loss of epidermal cells that are sloughed off in response to parasites (Wells and Cone, 1990). Unlike exposure to Zn alone, infection alone had minimal impact on mucin composition, despite reports that

more acidic mucins are produced in response to gyrodactylids on sticklebacks (Lester, 1972).

Between day 6 and day 18, combined exposure to Zn and gyrodactylid infection induced some responses that were similar to reactions seen to Zn alone (fluctuating epidermal thickness at concentrations higher than 15µg Zn/L) whereas others that were typical of responses to the parasite (decreased number of mucous cells throughout the infection). In response to the combined stresses, cells with mixed neutral and acidic mucins predominated, especially at concentrations of 15, 30 and 120µg Zn/L. Our data on epidermal thickness indicate that both Zn and infection influenced the host response, with Zn exposure contributing to the fluctuations in epidermal thickness especially at higher zinc concentrations and infection leading to an overall thickening of the epidermis (Fig. 5.2I, J), even beyond that seen in response to infection alone (Fig. 5.2F). In fact, when both stresses were combined, the thickness of the epidermis never dropped below control values, which it did in fish exposed to Zn alone. Epidermal thickening and associated hyperplasia has been reported as a general adaptive response to different stressors (Whitear, 1986; Wells and Cone, 1990; Iger *et al*, 1994; Sterud *et al.*, 1998). Perhaps the most intriguing observations were obtained for infected fish exposed to 60 µg Zn/L. Although epidermal thickness was higher throughout infection than for any other concentration, and although the number of mucous cells was lower, both typical responses to infection, 60 µg Zn/L was the only concentration where infected fish produced acidic mucins, a typical response to Zn (Fig. 5.4D, I). At all other concentrations, however, in the fish concurrently exposed to Zn and infection, the

response profile was apparently dominated by infection rather than Zn during days 6 through 18 as evidenced by sustained depression of mucous cell numbers and absence of shift in mucin composition. This is completely understandable given that parasite numbers are increasingly exponentially during this period whereas Zn concentrations are remaining stable.

Regardless of concentration, exposure to Zn for more than 18 days induced depressed mucous production and mucous cell numbers in fish, perhaps reflecting either acclimation to Zn, or exhaustion of the epidermal tissue due to inability of sustained differentiation of progenitor cells. Physiological acclimation to sublethal, chronic waterborne Zn has been reported, for example in trout where pre-exposure to Zn for a 5 day period increased the $LC_{50}$ by 2.5 times, compared to fish not pre-exposed to Zn (Bradley, DuQuesnay & Sprague, 1985). Acclimation occurs after an initial period of physical damage and disruption of physiological homeostasis, followed by a recovery period where tissue repair begins and synthesis of metal transport proteins is up-regulated, thus re-establishing a homeostatic equilibrium with increased tolerance to the metal (McGeer, Szebedinszky, McDonald & Wood, 2000). Our experimental design precluded us from examining the longer-term responses to a combination of Zn and infection.

Taken together, our present results showed that fish epidermis responded to both Zn exposure and gyrodactylid infection and the responses differed with concentration of Zn, duration of exposure and intensity of infection. Initial responses were most likely protective against both Zn toxicity (through increased mucous production, with more acidic composition), and infection (increased

epidermal thickness and). Although mucous is critically important in protecting the fish epidermis against entry of waterborne Zn, fish also use the epidermis and scales as a storage tissue for Zn (Sauer and Watabe, 1984, 1989; Nakano, Ono & Takeuchi, 1992). Both processes may contribute to the high tolerance of guppies to Zn (Widianarko *et al.*, 2000, 2001), as we recorded in guppies exposed to Zn alone. As concentration of Zn and parasite numbers increased, the epidermal responses indicated disturbed host response (dramatic decline in mucous cell numbers, with mixed composition of mucins), which became less effective against Zn toxicity and infection. Combined damage induced by both aggressors could account for the concentration-dependent elevation in mortality previously recorded in infected guppies concurrently exposed to Zn (Gheorghiu *et al.*, 2006). Thus as parasite numbers increased, the parasite-induced damage to the epidermis facilitated entry of Zn into inner tissues. In addition, as concentration of Zn increased, it exceeded the ability of mucous release, epithelial cells and scales to accumulate Zn, thus allowing entrance of excessive Zn into internal body structures and causing toxicity in a direct dose-dependent manner (Köck and Bucher, 1997; Widianarko *et al.*, 2000, 2001).

In conclusion, these results indicate that when exposed to both Zn and *Gyrodactylus* infection, fish have an impaired epidermal response, which on one hand will not be able to limit or clear the infection, and on the other hand, will no longer prevent the absorption of Zn into the body. Moreover, when combining two stressors, due to unexpected interactive effects, results are not necessarily linear. This is particularly important in interpreting physiological and toxicity

responses in the field when organisms are subjected to not only pollutants, but pathogens as well.

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## References


Appleby, C., Mo, T. A. & Aase, I. L., 1997. The effect of *Gyrodactylus salaris* (Monogenea) on the epidermis of Atlantic salmon, *Salmo salar*, parr in the river Batnfjordselva, Norway. *Journal of Parasitology* **83**, 1173-1174.

Bakke, T. A., Harris, P. D., Hansen, L. P. & Jansen, P. A., 1992. Host specificity and dispersal strategy in gyrodactylid monogenean with particular reference to *Gyrodactylus salaris* (Plathyhelminthes, Monogenea). *Diseases of Aquatic Organisms* **13**, 45-57.

Bancroft, J. D. & Stevens, A., 1982. *Theory and Practice of Histological Techniques*, 2nd ed., pp. 194-198, Churchill Livingstone, NY.

Barker, D. E., Cone, D. K. & Burt, M. D. B., 2002. *Trichodina murmanica* (Ciliophora) and *Gyrodactylus pleuronecti* parasitizing hatchery-reared winter flounder, *Pseudopleuronectes americanus* (Walbaum): effects on host growth and assessment of parasite interaction. *Journal of Fish Diseases* **25**, 81– 89.

Benedetti, I., Albano, A. G. & Mola, L., 1989. Histomorphological changes in some organs of the brown bullhead, *Ictalurus nebulosus* LeSueur, following short- and long-term exposure to copper. *Journal of Fish Biology* **34**, 273–280.

Bradley, R. W., DuQuesnay, C. & Sprague, J. B., 1985. Acclimation of rainbow trout, *Salmo gairdneri* Richardson, to zinc: kinetics and mechanism of enhanced tolerance induction. *Journal of Fish Biology* **27**, 367-379.

Buchmann, K.. 1999. Immune mechanisms in fish skin against monogeneans – a model. *Journal of Parasitology* **46**, 1-9.

Buchmann, K. & Bresciani, J., 1998. Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*: association between mucous cell density in skin and site selection. *Parasitological Research* **84**, 17-24.

Buchmann, K. & Bresciani, J., 1999. Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini. Diseases of Aquatic Organisms* **35**, 13-22.

Buchmann K. & Lindenstrøm T., 2002. Interactions between monogenean parasites and their fish hosts. *International Journal for Parasitology* **32,** 309-319.

Cable, J., Harris, P. D. & Tinsley, R.C., 1996. Ultrastructural adaptations for viviparity in the female reproductive system of gyrodactylid monogeneans. *Tissue and Cell* **28**, 515-526.

Canadian Council on Animal Care, 2005. Guidelines on: the care and use of fish in research, teaching and testing. http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Fish/Fish%20Guidelines%20English.pdf

Canadian Council of Ministers of the Environment, 2005. Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life. http://www.ccme.ca/assets/pdf/wqg_aql_summary_table.pdf

Cone, D. K., 1999. Monogenea. In *Fish Diseases and Disorders. Vol.1. Protozoan and Metazoan Infections.* (ed. Woo, P. T. K.), pp. 289-327. CABI Publishing, Wallingford, UK.

Gheorghiu, C., Cable, J., Marcogliese, D. J. & Scott, M. E., 2007 Effects of waterborne zinc on reproduction, survival and morphometrics of

*Gyrodactylus turnbulli* (Monogenea) on guppies (*Poecilia reticulata*). *International Journal for Parasitology* **37**, 375-381.

Gheorghiu, C., Marcogliese, D. J. & Scott, M., 2006. Concentration-dependent effects of waterborne zinc on population dynamics of *Gyrodactylus turnbulli* (Monogenea) on isolated guppies (*Poecilia reticulata*). *Parasitology* **132**, 225-232.

Handy, R. D., Eddy, F. B. & Romain, G., 1989. *In vitro* evidence for the ionoregulatory role of rainbow trout mucus in acid, acid/aluminium and zinc toxicity. *Journal of Fish Biology* **35**, 737-747.

Harris, P. D., Cable, J., Tinsley, R. C. & Lazarus, C. M., 1999. Combined ribosomal DNA and morphological analysis of individual gyrodactylid monogeneans. *Journal of Parasitology* **85**, 188-191.

Harris, P. D., Soleng, A. & Bakke, T. A., 1998. Killing of *Gyrodactylus salaris* (Platyhelminthes, Monogenen) mediated by host complement. *Parasitology* **117**, 137-143.

Iger, Y. & Wendelaar Bonga, S. E., 1993. Cellular responses of the skin of carp (*Cyprinus carpio*) exposed to acidified water. *Cell & tissue research* **275**, 481-492.

Iger, Y., Abraham M., Dotan A., Fattal B. & Rahamim E., 1988. Cellular responses in the skin of carp maintained in organically fertilized water. *Journal of Fish Biology* **33**, 711–720.

Iger, Y., Jenner, H. & Wendelaar Bonga, S. E., 1994. Cellular responses in the skin of rainbow trout (*Oncorhynchus mykiss*) exposed to Rhine water. *Journal of Fish Biology* **45**, 1119-1132.

Kearn, G. C., 1998. *Parasitism and the Platyhelminths*. London, (ed. by Chapmann and Hall), pp. 104-12.

Khan, R. A. & Kiceniuk, J. W., 1988. Effect of petroleum aromatic hydrocarbons on monogeneids parasitizing Atlantic cod, *Gadus morhua* L. *Bulletin of environmental contamination and toxicology* **41**, 94-100.

Köck, G. & Bucher, F., 1997. Accumulation of zinc in rainbow trout (*Oncorhynchus mykiss*) after waterborne and dietary exposure. *Bulletin of environmental contamination and toxicology* **58**, 305-10.

Lester, R. J. G., 1972. Attachment of *Gyrodactylus* to *Gasterosteus* and host response. *Journal of Parasitology*, **58**, 717 722.

Lester, R. J. G. & Adams, J. R., 1974. *Gyrodactylus alexandri*: reproduction, mortality, and effect on its host *Gasterosteus aculeatus*. *Canadian Journal of Zoology* **52**, 827-833.

McGeer, J. C., Szebedinszky, C., McDonald, D. G. & Wood, C. M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1. Iono-regulatory disturbance and metabolic costs. *Aquatic Toxicology* **50**, 231-234.

Nakano, T., Ono, K. & Takeuchi, M., 1992. Levels of zinc, irons, and copper in the skin of abnormally pigmented Japanese flounder. *Bulletin of the Japanese Society of Scientific Fisheries* **58**:2207.

Poléo, A. B. S., Schjolden, J., Hansen, H., Bakke, T. A., Mo, T. A., Rosseland, B. O. & Lydersen, E., 2004. The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology* **128**, 169-177.

Richards, G. R. & Chubb, J. C., 1996. Host response to initial and challenge infections, following treatment, of *Gyrodactylus bullatarudis* and *Gyrodactylus turnbulli* (Monogenea) on the guppy *(Poecilia reticulata). Parasitological Research* **82**, 242-247.

Richards, G. R. & Chubb, J. C., 1998. Long-term population dynamics of *Gyrodactylus bullatarudis* and *G. turnbulli* (Monogenea) on adult guppies (*Poecilia reticulata*) in 50-L experimental arenas. *Parasitological Research* **84**, 753-756.

Roberts, R. J. & Bullock, A. M., 1980. The skin surface ecosystem of teleost fishes. *Proceedings of the Royal Society of Edinburgh* **79B**, 87-91.

Sauer, G. R. & Watabe, N., 1984. Zinc uptake and its effect on calcification in the scales of the mummichog, *Fundulus heteroclitus. Aquatic Toxicology* **5**, 51-66.

Sauer, G. R. & Watabe, N., 1989. Ultrastructural and histochemical aspects of zinc accumulation by fish scales. *Tissue and Cell* **21**, 935-943.

Scott, M. E., 1982. Reproductive potential of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* **85**, 217-236.

Scott, M. E., 1985. Dynamics of challenge infections of *Gyrodactylus bullatarudis* Turnbull (Monogenea) on guppies, *Poecilia reticulata* (Peters). *Journal of Fish Diseases* **8**, 495-503.

Scott, M. E. & Anderson, R. M., 1984. The population dynamics of *Gyrodactylus bullatarudis* (Monogenea) within laboratory populations of the fish host *Poecilia reticulata*. *Parasitology* **89**, 59-94.

Scott, M. E. & Nokes, D. J., 1984. Temperature-dependent reproduction and survival of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* **89**, 221-227.

Scott, M. E.& Robinson, M. A., 1984. Challenge infections of *Gyrodactylus bullatarudis* (Monogenea) on guppies, *Poecilia reticulata* (Peters), following treatment. *Journal of Fish Biology* **24**, 581-586.

Shephard, K. L., 1994. Functions for fish mucus. *Reviews in Fish Biology and Fishereis* **4**, 401-29.

Sinha, G. M. & Chakravorty, P., 1982. Characterization and distribution of neutral and acidic mucins in the alimentary canal of an Indian freshwater major carp, *Catla catla* (Hamilton) by histochemical methods. *Gegenbaurs Morphologisches Jahrbuch* **128**, 188-200.

Soleng, A., Poléo, A. B. S., Alstand, N. E. W. & Bakke, T. A., 1999. Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology* **119**, 19-25.

Sterud, E., Harris, P. D. & Bakke, T. A., 1998. The influence of *Gyrodactylus salaris* Malmberg 1957 (Monogenea) on the epidermis of Atlantic salmon, *Salmo salar* L., and brook trout, *Salvelinus fontinalis* (Mitchill): experimental studies. *Journal of Fish Diseases* **21**, 257-263.

Tibbetts, I. R., 1997. The distribution of mucous cells and their secretions in the alimentary tract of *Arrhamphus sclerolepis*. *Journal of Fish Biology* **50**, 809-820.

Triebskorn, R., Christensen, K.& Heim, I., 1998. Effect of orally and dermally applied metaldehyde on mucus cells of slugs *Deroceras reticulatum* depending on temperature and duration of exposure. *Journal of Molluscan Studies* **64**, 467-487.

Wells, P. R. & Cone, D. K., 1990. Experimental studies on the effect of *Gyrodactylus colemanensis* and *G. salmonis* (Monogenea) on density of mucous cells in the epidermis of fry of *Oncorhynchus mykiss*. *Journal of Fish Biology* **37**, 599-603.

Whitear, M., 1986. Epidermis. In *Biology of the Integument. 2. Vertebrates* (ed. Bereiter-Hahn J, Matoltsy AG and Richards KS), pp.8-38. Berlin, Springer-Verlag.

Widianarko, B., Kuntoro, F. X. S., van Gestel, C. A. M., Verweij, R. A. & van Straalen, N. M., 2001. Toxicokinetics and toxicity of zinc under time-varying exposure in the guppy (*Poecilia reticulata*). *Environmental Toxicology and Chemistry* **20**, 763-768.

Widianarko, B., van Gestel, C. A. M., Verweij, R. A. & van Straalen, N. M., 2000. Associations between trace metals in sediment, water, and guppy, *Poecilia reticulata* (Peters), from urban streams of Semarag, Indonesia. *Ecotoxicology and Environmental Safety* **46B**, 101-107.

# CHAPTER 6

# GENERAL DISCUSSION

This research emphasized the complex effects of Zn on the interactions between *G. turnbulli* and their host guppies. As with all research, although some answers have been found, a wide range of new questions have emerged.

One of our major findings is increasing mortality with the increase of waterborne Zn concentration, but only in infected fish. Why does mortality of fish exposed to sublethal concentrations of Zn occur only in the presence of infection? We attributed the linear increase in mortality with increasing Zn concentrations to the combined effect of Zn exposure and infection. On one hand, the parasite is known to disrupt the integrity of the epidermis while moving and feeding on the skin surface, thus creating entry points for potential secondary infections (Kearn, 1998; Cone, 1999) and for Zn. On the other hand, the key factor in the host response against both Zn and parasite is increased mucous production that prevents entry of Zn into the inner tissues and that clears the infection (Shephard, 1994). However, data from our third set of experiments (Chapter 5, Gheorghiu *et al.*, submitted) indicate that the protective effect of mucus was lost, because the ability of the host to secrete mucus was apparently exceeded as both concentration of Zn and parasite numbers increase. An additional mechanism to prevent Zn toxicity is represented by its accumulation in the storage tissues. Epithelial cells and scales are reported as important for Zn storage (Sauer and Watabe, 1984, 1989; Nakano *et al.*, 1992). As the

concentration of Zn increased, it is also possible that the physical damage caused by infection impaired the ability of epithelial cells and scales to accumulate Zn and the excessive Zn eventually reached the internal body structures, damaging them and disrupting the ionic balance. Both limited storage capacity and exhaustion of mucus production would increase the susceptibility of guppies to Zn toxicity. To test the hypothesis of increased accumulation of Zn in the tissues of infected fish, future research is needed. Given the small size of the guppy, it would be better if such research were done on a different host-*Gyrodactylus* system, using a bigger size species of fish. Alternatively, as new fluorescent markers will become available, it would be helpful to detect the histological differences in Zn-accumulation in various fish structures as the concentration of Zn increases. This method could be used in guppies.

Buchmann and Bresciani (1998) showed that fish epithelium and mucus contain complement and concluded that gyrodactylids live and feed in an environment rich complement factors. Several *in vitro* studies have indicated that the complement system present in mucus is important for parasite killing (Buchmann, 1998; Buchmann and Bresciani, 1998; Harris *et al.*, 1998; 2000), and that activated macrophages in fish skin may be the primary source of the complement (Buchmann and Bresciani, 1999). Interestingly, Sanchez-Dardon *et al.* (1999) showed suppression of phagocytic activity of macrophages in rainbow trout exposed to 10 - 50 µg Zn/L for 30 days; thus Zn may also suppress other functions of macrophages as well, including activation of the complement. So, exposure of infected fish to waterborne Zn might impair complement activation, and thus favor the parasite population growth as reported in our first study

(Chapter 3; Gheorghiu *et al.*, 2006). Using current technology, further exploration of the interaction between Zn and complement in infected fish should be pursued in a different host – *Gyrodactylus* system, using a host species large enough to enable collection of mucus and plasma.

Although Zn is toxic to infected fish, it is also toxic to the parasite, as proven by reduced survival and morphometrics in response to Zn (Chapter 4; Gheorghiu *et al.*, in press). Disentangling direct and indirect effects of Zn and any parasite is difficult given the intimate relationship between the host and parasite. *Gyrodactylus* is no different. In fact as an ectoparasite, it is exposed directly to Zn in water and to the host response elicited in response to both infection and Zn in the water. Moreover, as *Gyrodactylus* bears in its uterus several embryos one inside another, it is possible that cumulative effects of Zn exposure affect the subsequent generations. Due to the unique biology of *Gyrodactylus* (viviparity, direct life cycle, attachment of the new-born individuals beside their mothers on the same host), in order to understand the mechanism of Zn toxicity to the parasite, we felt it was important to separate as far as possible the additional effects induced by host response to infection and/or Zn exposure, and by density pressure during the exponential phase of the infection. Thus, to minimize the effects of host response and parasite density on parasite reproduction and lifetime survival, we designed experiments so that infected fish only harbored one or two parasites at a time. As such, the survival and reproduction of parasites on the fish was only affected by direct Zn toxicity to parasites and host response induced by Zn exposure. If exponential growth of the parasite had occurred, the effects on *Gyrodactylus* survival and reproduction would have been further

complicated by host response to infection and by density pressure of the increasing population of worms. In its turn, the density pressure could impair parasite survival by inducing a stronger host response.

Further, given that parasites can become detached from their hosts and are able to survive for short periods of time and reattach to another host, it is important to understand the direct impact of waterborne Zn on isolated parasites from the host. To pull apart the direct effect of Zn on parasite survival from indirect effects induced through the host response, the parasites were isolated from the host and exposed individually to waterborne Zn. Direct toxicity of Zn to the parasites was best shown by the decreasing survival of the detached parasites as the concentration of Zn increased. Our results were similar to those obtained by Morley *et al.* (2001), who reported reduced survival of *Schistosoma mansoni* miracidia exposed to 100 µg Zn/L and higher. In addition, Morley *et al.* (2003) recorded impaired host location behaviour on *Echinoparyphium recurvatum* cercariae exposed to concentrations as low as 10 µg Zn/L. They attributed this result to Zn-selective binding to sensory structures involved in sensorial host location and infection. It is possible that exposure of detached parasites to Zn reduces *Gyrodactylus* re-attachment to new hosts as well, with negative implications for parasite transmission in the host population. This hypothesis could be tested by using Zn-exposed parasites to initiate experimental infection, or to assess their rate of transmission to new fish. Furthermore, future studies should address Zn-accumulation in parasite structures in order to examine if a similar mechanism would be involved in impaired host location.

Toxic effects of waterborne Zn exposure to *G. turnbulli* were also shown by the linear decrease of morphometrics of exposed parasites as the concentration of Zn and duration of exposure increased. Although previous research on parasite morphometrics in response to different environmental factors (i.e. temperature) focused on hard parts of the opisthaptor (Geets *et al.*, 1999), when designing the experiment, we believed that the soft-body parts would respond more quickly to environmental zinc than the hard parts. Given that all the morphometrics decreased with increasing concentration of Zn and duration of exposure, it would be possible that the same effect to be extended to the hard parts of the opisthaptor too. However, as our primary interest was in the survival and reproduction of the parasite, we considered it more biologically relevant to quantify changes in soft body parts. Also, in our experience, it is more difficult to get accurate measurements of hooks and hamuli in fresh specimens than of soft body parts. The recommended techniques for measuring hooks and hamuli require destruction of the worms and their internal organs, or digestion of soft tissues (Harris, 1986; Shinn *et al.*, 1993; Cable *et al.*, 1998). Thus we would not have been able to measure both soft and hard tissues in the same individuals.

Given that these decreases in size in response to Zn and duration of exposure were recorded during mini-epidemics of infected fish exposed to Zn where parasite exponential growth and transmission between hosts was allowed, we attributed our results to a change in the age structure of the parasites population (Chapter 4; Gheorghiu *et al.*, 2007). Although *Gyrodactylus* are considered fully-developed immediately at birth, some structures develop later on, potentially affecting the parasite morphometrics, but this remains to be tested.

Depending on the development of embryos inside the uterus and the occurrence of male reproductive apparatus, *Gyrodactylus* individuals can be categorized in different age classes (Cable *et al.*, 2002). Moreover, the development of the embryos inside the uterus affects the width of the parasites. So, the age distribution of individual worms of a population of *Gyrodactylus* depends on the instantaneous birth rate and the instantaneous death rate, which are age dependent (Scott, 1982). In addition, *Gyrodactylus* survival and reproduction is strongly influenced by environmental factors, including waterborne Zn (Chapter 4; Gheorghiu *et al.*, 2007). So, further research to detect the impact of Zn exposure on the parasite population age structure should be done using either isolated hosts or mini-populations of fish.

Across all our experiments, waterborne Zn in concentrations of 60 µg/L induced the most unpredictable, counter-intuitive responses. Thus, unlike in detached parasites exposed to Zn, Zn was only toxic to attached parasites exposed to 30 and 120 µg Zn/L, but surprisingly, not to 60 µg Zn/L (Chapter 4; Gheorghiu *et al.*, 2007). In addition, after an initial release of mucus in response to waterborne Zn exposure, fish exposed to 60 µg Zn/L did not produce mucus between days 6 and 15 post exposure, whereas the fish exposed to all other experimental Zn concentrations responded with more rapid fluctuations in mucus release. Histologically, the response recorded in fish exposed to 60 µg Zn/L corresponded to a more marked decrease in mucous cell numbers and a shift to acidic mucin composition, when compared with the other concentrations. Moreover, in infected fish exposed to 60 µg Zn/L, we recorded the highest epidermal thickness, more marked depletion in mucous cell numbers and the

same shift to more acidic composition as in uninfected fish exposed to the same concentration of Zn (Chapter 5; Gheorghiu *et al*, submitted). Similar non-linear dose response patterns have been recorded in other systems when more than two factors affect simultaneously the system taken in consideration (Sih *et al.*, 2004). Our results could be explained by an impaired host response against the parasite, which no longer limits the parasite exponential growth in fish exposed to 60 µg Zn/L, combined with a reduced toxicity of Zn to the parasite. To obtain a better understanding of the mechanisms, more research is required on specific host response to infection in fish exposed to 60 µg Zn/L, and on Zn toxicity to the parasite.

The present research demonstrated that waterborne Zn, suggested to be at sublethal concentrations to guppy (Eisler, 1993), affected both host and parasite, but was more detrimental to the infected hosts than to the parasite. These results are particularly important in aquaculture and fisheries, given that *Gyrodactylus* spp. affect almost all species of teleost fish, and moreover, Zn pollution is one of the most commonly encountered (Bowen *et al.*, 2006). In addition, recent research on Atlantic salmon (*Salmo salar*) infected with *G. salaris*, the most deadly species, which devastated natural salmon population in many infected rivers in Norway (Johnsen and Jensen, 1991; Bakke *et al.*, 1992; 2000), focused on potential use of waterborne Zn and other heavy metals as a possible method for treating infection in the wild. Even though their results are promising, as the parasite was apparently more affected than the host, proper caution should be taken, especially because *Salmo salar* is more sensitive to waterborne Zn than guppies. Thus, whereas $LC_{50}$ (96h) for immature Atlantic

salmon is 420 - 600 µg Zn/L, depending on the water hardness, the same indicator for guppies is 1350-1500 µg Zn/L for fry, and 4400-7300 µg Zn/L for adults, depending on water hardness and fish gender (Eisler, 1993).

The Canadian Water Quality Guidelines (2005) stipulate that aqueous Zn should not exceed 30 µg Zn/L for aquatic life. Our results showed that at this concentration, toxicity to fish increases when they are infected. Thus, if 30 µg Zn/L is the cut off for uninfected fish, it may be too high for infected fish, in which we recorded epidermal changes (Chapter 5; Gheorghiu *et al.*, submitted) and mortality (Chapter 3; Gheorghiu *et al.*, 2006). Whereas exposure to 30 µg Zn/L alone did not induce any mortality in the first 35 days of exposure, the same parameter measured in infected fish exposed to the same concentration of Zn, increased to 54% mortality (Chapter 3; Gheorghiu *et al.*, 2006). In addition, mortality in infected unexposed fish was 24 %, but when infected fish were exposed to even the lowest concentration of Zn (15 µg Zn/L), mortality more than doubled (52%). In conclusion, to consider a certain concentration of a pollutant as an admissible limit, research needs to consider the sensitivity to the toxicant of both uninfected and infected individuals.

**References**

Bakke, T.A., Harris, P.D. and Cable, J., 2000. Host specificity dynamics: observations on gyrodactylid monogeneans. *Int. J. Paras.* 32: 281-308.

Bakke, T.A., Harris, P.D., Hansen, L.P. and Jansen, P.A., 1992. Host specificity and dispersal strategy in gyrodactylid monogenean with particular reference to *Gyrodactylus salaris* (Platyhelminthes, Monogenea). *Dis Aquat. Org.* 13: 45-57.

Bowen, L., Werner, I., and Johnson, M.L., 2006. Physiological and behavioural effects of zinc and temperature on coho salmon (*Oncorhynchus kisutch*). *Hydrobiologia* 559: 161-168.

Buchmann, K., 1998. Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). *Dis. Aquat. Org.* 32: 195-200.

Buchmann, K. and Bresciani, J., 1998. Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*: association between mucous cell density in skin and site selection. *Parasitol. Res.* 84: 17-24.

Buchmann, K. and Bresciani, J., 1999. Rainbow trout leukocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini. Dis. Aquat. Org.* 35: 13-22.

Cable, J., Harris, P.D. and Tinsley, R.C., 1998. Life history specializations of monogenean flatworms: a review of experimental and microscopical studies. *Microsc. Res.Tech.* 42: 186-199.

Cable, J., Tinsley, R.C. and Harris, P.D., 2002. Survival, feeding and embryo development of *Gyrodactylus gasterostei* (Monogenea: Gyrodactylidae). *Parasitology* 124: 53-68.

Canadian Council of Ministers of the Environment, 2005. Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life. http://www.ccme.ca/assets/pdf/wqg_aql_summary_table.pdf

Cone, D.K., 1999. Monogenea. In *Fish Diseases and Disorders. Vol. 1. Protozoan and Metazoan Infections* (ed. Woo, P.T.K.), pp. 289-327. CABI Publishing, Wallingford, UK.

Eisler, R., 1993. Zinc hazards to fish, wildlife and invertebrates: a synoptic review. U.S. Department of the Interior Fish and Wildlife Service. Patuxent Wildlife Research Center Biological Report 10. Contaminant Hazard Reviews Report 26. Laurel, Maryland 20708.

Geets, A., Appleby, C., and Ollevier, F., 1999. Host-dependent and seasonal variation in opisthaptoral hard parts of *Gyrodactylus* cf. *arcuatus* from three *Pomatoschistus* spp. and *G. arcuatus* from *Gasterosteus aculeatus*: a multivariate approach. *Parasitology* 119: 27-40.

Gheorghiu, C., Cable, J., Marcogliese, D.J. and Scott, M.E., 2007 Effects of waterborne zinc on reproduction, survival and morphometrics of *Gyrodactylus turnbulli* (Monogenea) on guppies (*Poecilia reticulata*). *Int. J. Parasitol.* 37(3-4): 375-381.

Gheorghiu, C., Marcogliese, D.J., and Scott M.E. Dynamics of epidermal responses of guppies (*Poecilia reticulata*) to two external stressors: waterborne zinc and *Gyrodactylus turnbulli* (Monogenea) infection. *J.Fish Dis.* (submitted).

Gheorghiu, C., Marcogliese, D.J. and Scott, M., 2006. Concentration-dependent effects of waterborne zinc on population dynamics of *Gyrodactylus turnbulli* (Monogenea) on isolated guppies (*Poecilia reticulata*). *Parasitology* 132: 225-232.

Harris, P.D., 1986. Species of *Gyrodactylus* von Normann 1832 (Monogenea, Gyrodactylidae) from poeciliid fishes with a description of *G. turnbulii* sp.n. from the guppy *Poecilia reticulata* (Peters). *J. Nat.History* 20: 183-191.

Harris, P.D., Soleng, A. and Bakke, T.A., 1998. Killing of *Gyrodactylus salaris* (*Platyhelminthes, Monogenean*) mediated by host complement. *Parasitology* 117: 137-143.

Harris, P.D., Soleng, A. and Bakke, T.A., 2000. Increased susceptibility of salmonids to the monogenean *Gyrodactylus salaris* following administration of hydrocortisone acetate. *Parasitology* 120: 57-64.

Johnsen, B.O. and Jensen, A.J., 1991. The *Gyrodactylus* story in Norway. *Aquaculture.* 98: 289-302.

Kearn, G.C., 1998. *Parasitism and the Platyhelminths.*, London, ed. Chapmann and Hall, pp. 104-112.

Morley, N.J., Crane, M. and Lewis, J.W., 2001. Toxicity of cadmium and zinc to miracidia of *Schistosoma mansoni*. *Parasitology* 122: 81-85.

Morley, N.J., Crane, M. and Lewis, J.W., 2003. Effects of cadmium and zinc toxicity on orientation behaviour of *Echinoparyphium recurvatum* (Trematoda: Echinostomidae) cercariae. *Dis. Aquat. Org.* 56: 89-92.

Nakano, T., Ono, K. and Takeuchi, M., 1992. Levels of zinc, irons, and copper in the skin of abnormally pigmented Japanese flounder. *Bull. Jpn. Soc. Sci. Fish.* 58: 2207.

Sanchez-Dardon, J., Voccia, I., Hontela, A., Chilmonczyk, S., Dunier, M., Boermans, H., Blakley, B. and Fournier, M., 1999. Immunomodulation by heavy metals tested individually or in mixtures in rainbow trout (*Oncorhynchus mykiss*) exposed *in vivo*. *Environ. Toxicol. Chem.* 18: 1492–1497.

Sauer, G.R. and Watabe, N., 1984. Zinc uptake and its effect on calcification in the scales of the mummichog, *Fundulus heteroclitus*. *Aquat. Toxicol.* 5: 51-66.

Sauer, G.R. and Watabe, N., 1989. Ultrastructural and histochemical aspects of zinc accumulation by fish scales. *Tis. Cell* 21: 935-943.

Scott, M.E., 1982. Reproductive potential of *Gyrodactylus bullatarudis* (Monogenea) on guppies (*Poecilia reticulata*). *Parasitology* 85: 217-236.

Scott, M.E. and Anderson, R.M., 1984. The population dynamics of *Gyrodactylus bullatarudis* (Monogenea) within laboratory populations of the fish host *Poecilia reticulata*. *Parasitology* 89: 159-194.

Shephard, K.L., 1994. Functions for fish mucus. *Rev Fish Biol. Fish.* 4: 401-429.

Shinn, A.P., Gibson, D.I. and Sommerville, C., 1993. An SEM study of the haptoral sclerites of the genus *Gyrodactylus* Nordmann, 1832 (Monogenea) following extraction by digestion and sonication techniques. *Syst. Parasitol.* 25: 135–144.

Sih, A., Bell, A. M. and Kerby, A. J. L., 2004. Two stressors are far deadlier than one. *Trends in Ecology and Evolution*, 19: 274-276.

# APPENDIX A

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**E-mail received from Marc P. Anderson, email: manderson@cambridge.org, on January 17, 2007 (AH: PF)**

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Concentration-dependent effects of waterborne zinc on population dynamics of Gyrodactylus turnbulli (Monogenea) on isolated guppies (Poecilia reticulata)
C. GHEORGIU, D. J. MARCOGLIESE and M. SCOTT
Parasitology, Volume 132, Issue 02, February 2006, pp 225-232

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pubyear = 2006
book = editor
bookauthor = Cambridge University Press
isbn =
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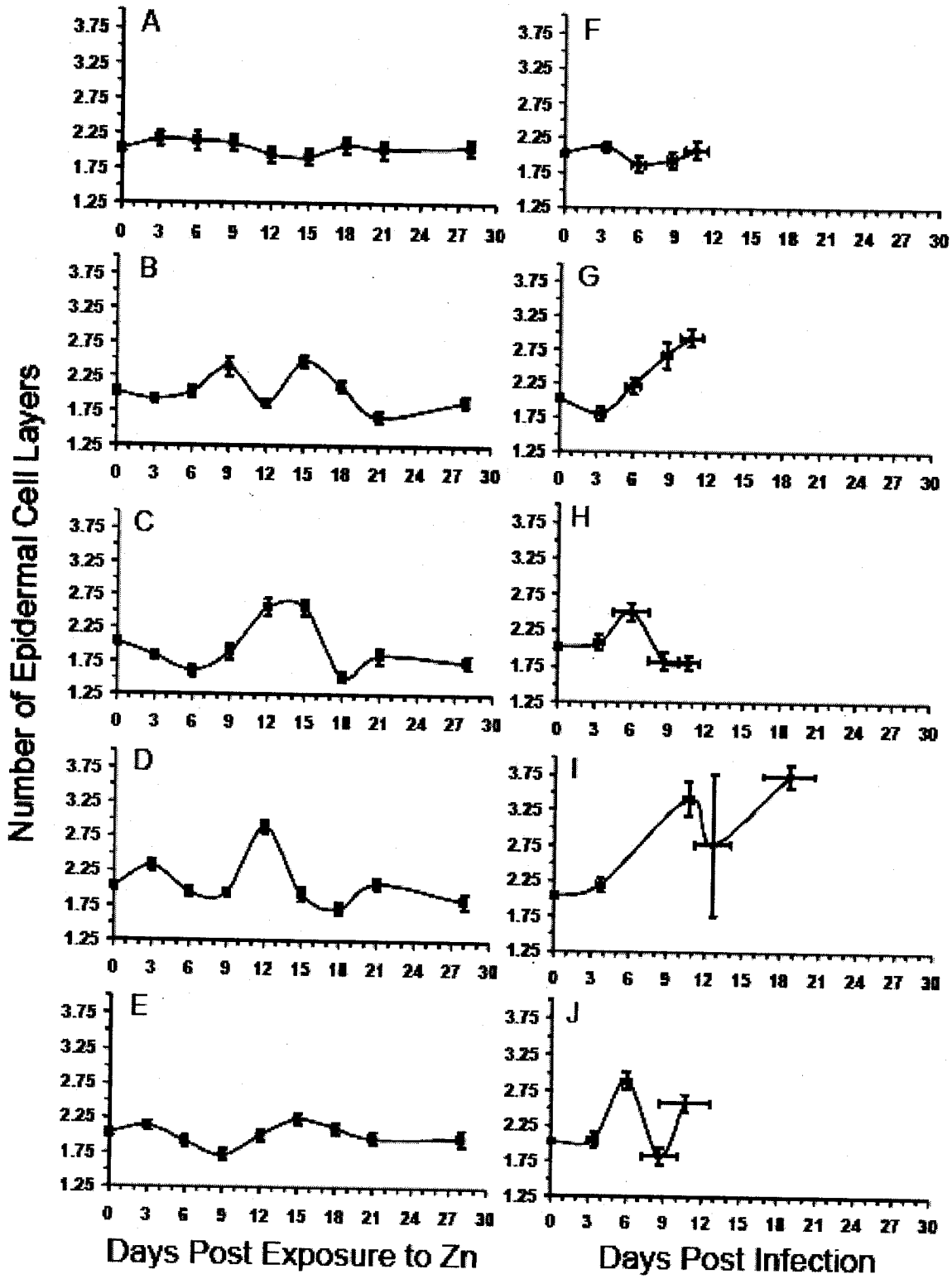
# APPENDIX C

**Temporal dynamics of number of epidermal cell layers in response to Zn and/or *Gyrodactylus*.**

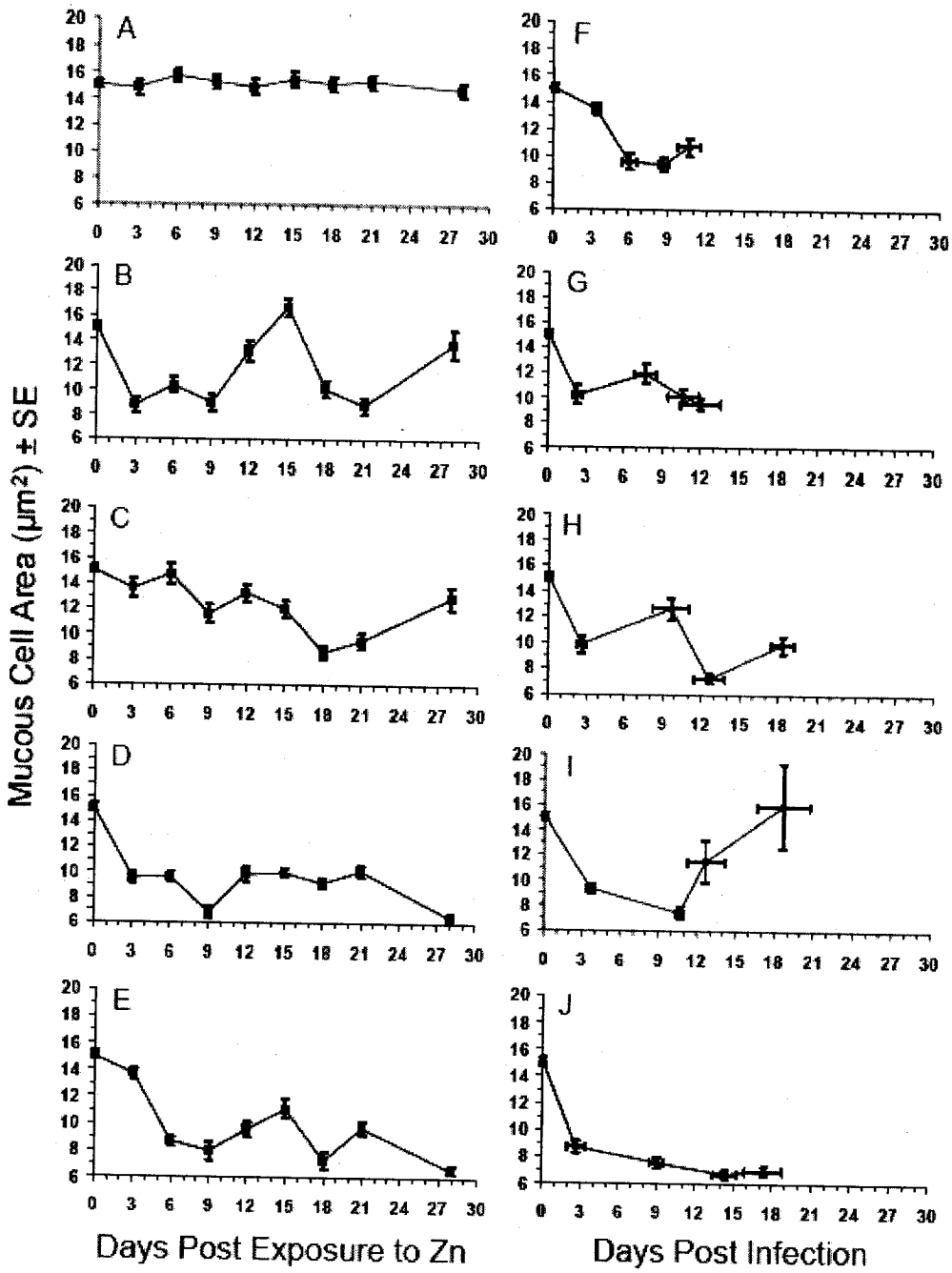
(A) 0 µg Zn/L; (B) 15 µg Zn/L; (C) 30 µg/L; (D) 60 µg Zn /L; (E) 120 µg Zn/L; (F) *Gyrodactylus*; (G) *Gyrodactylus* and 15 µg Zn/L; (H) *Gyrodactylus* and 30 µg Zn/L; (I) *Gyrodactylus* and 60 µg Zn/L; (J) *Gyrodactylus* and 120 µg Zn/L. Main effects: Zn, $p<0.0001$, time, $p<0.0001$, Zn*time, $p<0.0001$.


A
B
C
D
E
F
G
H
I
J
Number of Epidermal Cell Layers
Days Post Exposure to Zn
Days Post Infection

# APPENDIX D

**Temporal dynamics of mucous cell size ($\mu m^2$) in response to Zn and/or *Gyrodactylus*.**

(A) 0 µg Zn/L; (B) 15 µg Zn/L; (C) 30 µg/L; (D) 60 µg Zn /L; (E) 120 µg Zn/L; (F) *Gyrodactylus*; (G) *Gyrodactylus* and 15 µg Zn/L; (H) *Gyrodactylus* and 30 µg Zn/L; (I) *Gyrodactylus* and 60 µg Zn/L; (J) *Gyrodactylus* and 120 µg Zn/L. Main effects: Zn, $p<0.0001$, time, $p<0.0001$, Zn*time, $p<0.0001$.


A
B
C
D
E
F
G
H
I
J
Mucous Cell Area (µm²) ± SE
Days Post Exposure to Zn
Days Post Infection

# APPENDIX E

## Animal Use Protocol

Guidelines for completing this form are available at www.mcgill.ca/rgo/animal

# McGill University
## Animal Use Protocol – Research

For office use only
Protocol #: 4698.
Investigator #: 404
Approval End Date: Feb 28, 2004
Facility Committee: AGR

**Title:** Effect of water-borne zinc on *Gyrodactylus* infections on guppies
*(must match the title of the funding source application)*

**New Application:** X **Renewal of Protocol: #** **Pilot:** **Category** *(see section 11)*: B

**1. Investigator Data:**

**Principal Investigator:** Marilyn E. Scott **Phone #:** 398-7996
**Department:** Institute of Parasitology **Fax#:** 398-7857
**Address:** Macdonald Campus **Email:** Marilyn.scott@mcgill.ca

**2. Emergency Contacts:** Two people must be designated to handle emergencies.

| | | | | | |
|---|---|---|---|---|---|
| **Name:** | Gordon Bingham | **Work #:** | 398-7731 | **Emergency #:** | 457-9138 |
| **Name:** | Jim Smith | **Work #:** | 398-7726 | **Emergency #:** | 426-8870 |

**3. Funding Source:**

| | |
|---|---|
| **External:** NSERC | **Internal:** |
| **Source (s):** | **Source (s):** |
| **Peer Reviewed:** YES X NO** | **Peer Reviewed:** YES NO** |
| **Status:** Awarded Pending | **Status:** Awarded Pending |
| **Funding period:** | **Funding period:** |

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| | | |
|---|---|---|
| **Proposed Start Date of Animal Use (d/m/y):** | March 1, 2003 | **or ongoing:** |
| **Expected Date of Completion of Animal Use (d/m/y):** | Dec 31, 2004 | **or ongoing:** |

**Investigator's Statement:** The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

**Principal Investigator's signature:** Marilyn E Scott **Date:** 11 March 2003

*Approved by:*

| | | |
|---|---|---|
| **Chair, Facility Animal Care Committee:** | | **Date:** 11/3/03 |
| **University Veterinarian:** | | **Date:** 3/11/03 |
| **Chair, Ethics Subcommittee** *(as per UACC policy)*: | | **Date:** |
| **Approved Animal Use** | **Beginning:** MARCH 1, 2003 | **Ending:** Feb 28, 2004 |

This protocol has been approved with the modifications noted in Section 13.

May 2002

MAR 12 2003

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# McGill University
# Animal Use Protocol – Research

Protocol # 4698
Investigator # 404
Approval End Date: Feb 28, 2005
Facility Committee: AGR

**Title:** Effect of water-borne zinc on Gyrodactylus infections on guppies
*(must match the title of the funding source application)*

☐ New Application ☒ Renewal of Protocol # 4698 ☐ Pilot **Category** *(see section 11)*: C

## 1. Investigator Data:

**Principal Investigator:** Marilyn E. Scott **Phone #:** 398-7996
**Unit/Department:** Institute of Parasitology **Fax#:** 398-7857
**Address:** Macdonald Campus **Email:** marilyn.scott@mcgill.ca

## 2. Emergency Contacts: Two people must be designated to handle emergencies.

**Name:** Gordon Bingham **Work #:** 398-7731 **Emergency #:** (457) 9138
**Name:** Jim Smith **Work #:** 398-7726 **Emergency #:** (426) 8870

## 3. Funding Source:

**External** ☒
**Source (s):** NSERC
**Peer Reviewed:** ☒ YES ☐ NO**
**Status :** ☐ Awarded ☐ Pending
**Funding period:** 04/02-03/07

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**Funding period:** _____

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**Principal Investigator's signature:** Marilyn E Scott **Date:** 2 March 2004

*Approved by:*

| | | |
|---|---|---|
| **Chair, Facility Animal Care Committee:** | J McSmith | **Date:** 17 Mar 2004 |
| **University Veterinarian:** | J Shutya | **Date:** march 17, 2004 |
| **Chair, Ethics Subcommittee** (as per UACC policy): | | **Date:** |
| **Approved Animal Use** | **Beginning:** MARCH 1, 2004 | **Ending:** Feb 28, 2005 |

☒ This protocol has been approved with the modifications noted in Section 13.



19 MAR 2004

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# McGill University Animal Care Committee
# RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Protocol #: 4698
Approval end date: Feb 28, 2006
Facility Committee: AGR
Renewal#: (1st) 2nd

Principal Investigator: Marilyn Scott
Protocol # 4698
Protocol Title: Effect of water-borne zinc on Gyrodactylus infections on guppies
Phone: 398-7996
Unit, Dept. & Address: Parasitology, Macdonald Campus
Fax: 398-7857
Email: marilyn.scott@mcgill.ca
Level: B
Funding source: NSERC
Start of Funding: 04/02
End of Funding: 03/07
Emergency contact #1 + phone #s Gordon Bingham; work 398-7731; home 457-9138
Emergency contact #2 + phone #s Cristina Gheorghiu; work 398-8382; home 457-7068

CCAC
08 IV Mar 3 '05
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## 1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign. *(Space will expand as needed)*

| Name | Classification | Animal Related Training Information | Occupational Health Program * | Signature *"Has read the original full protocol"* |
|---|---|---|---|---|
| Marilyn Scott | Investigator | Workshop on Animal Care (WAC) Advanced Theory Course (ATC) | na | Marilyn E Scott Nov 3 |
| Cristina Gheorghiu | Grad Student | WAC, ATC (Basic & Wildlife) Experimental Fish - CAI | yes | 4R? |
| Gordon Binghman | Technician | WAC, ATC | yes | |

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## 2. Approval Signatures

| | | |
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| Principal Investigator/ Course Director | Marilyn E Scott | Date: 1 Feb 2005 |
| Chair, Facility Animal Care Committee | Linda Weiser | Date: Feb 20, 2005 |
| UACC Veterinarian | David Bainbridge | Date: Feb 25, 2005 |
| Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)* | — | Date: — |
| Approved Animal Use Period | Start: March 1, 2005 | End: Feb. 28, 2006 |

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The aim of this project is to determine how fish ectoparasites are affected by waterborne zinc. We hypothesize that zinc will accumulate in fish skin and impair the host response against the parasite, and as a consequence, fish will experience a greater mortality. In addition, we hypothesize that zinc will be harmful to the reproduction or morphology of the parasite, and as a consequence, zinc will act as a potential cure for the affected fish. Depending on the waterborne zinc concentration, the parasite population may be reduced or increased.

22 FEV. 2005

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# McGill University Animal Care Committee
# RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Protocol #: 4698
Approval end date: Feb 28, 2007
Facility Committee: AGR
Renewal#: 1st 2nd

**Principal Investigator:** Marilyn Scott — **Protocol #** 4698

**Protocol Title:** Effect of water-borne zinc on Gyrodacytlus infections on guppies / Host Parasite population Dynamics in Experimental Arenas

**Phone:** 398-7996

**Unit, Dept. & Address:** Parasitology, Macdonald Campus — **Fax:** 398-7857

**Email:** marilyn.scott@mcgill.ca — **Level:** B — **Funding source:** NSERC 18024

**Start of Funding:** 04/02 — **End of Funding:** 03/07

**Emergency contact #1 + phone #s** Gordon Bingham; work 398-7731; home 457-9138

**Emergency contact #2 + phone #s** Cristina Gheorghiu; work 398-8382; home 457-7068

## 1. Personnel and Qualifications

**List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)***

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|---|---|---|---|---|
| Marilyn Scott | Investigator | Workshop on Animal Care (WAC) Advanced Theory Course (ATC) | na | Marilyn Scott |
| Cristina Gheorghiu | Grad Student | WAC, ATC (Basic & Wildlife) Experimental Fish - CAI | yes | |
| Gordon Binghman | Technician | WAC, ATC | yes | |

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| | | |
|---|---|---|
| **Principal Investigator/ Course Director** | Marilyn Scott | **Date:** Feb 15, 2006 |
| **Chair, Facility Animal Care Committee** | Linda Wilkes | **Date:** Feb 27, 06 |
| **UACC Veterinarian** | Mabbye | **Date:** Nov 22, 2006 |
| **Chairperson, Ethics Subcommittee** *(D level or Teaching Protocols Only)* | | **Date:** |
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## 3. Summary (in language that will be understood by members of the general public)

**AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol).***

The aim of this project is to determine how fish ectoparasites are affected by waterborne zinc. We hypothesize that zinc will accumulate in fish skin and impair the host response against the parasite, and as a consequence, fish will experience a greater mortality. In addition, we hypothesize that zinc will be harmful to the reproduction or morphology of the parasite, and as a consequence, zinc will act as a potential cure for the affected fish. Depending on the waterborne zinc concentration, the parasite population may be reduced or increased.

# APPENDIX F

## Use of Biohazardous Materials

# McGill University

**University Biohazards Committee**

## APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Marilyn SCOTT

ADDRESS: Institute of Parasitology — TELEPHONE: 398-7996

Macdonald Campus — FAX NUMBER: 398-7857

DEPARTMENT: Institute of Parasitology — E-MAIL: marilyn.scott@mcgill.ca

PROJECT TITLE: Effect of water-borne zinc on *Gyrodactylus* infections on guppies

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2. FUNDING SOURCE: CIHR ☐ NSERC X☐ NIH ☐ FQRNT ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify)

Grant No.: A3585 Beginning date April 2002 End date March 2007

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3. Indicate if this is

☐ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency — Approval End Date

X New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): (1) 2 3 4

Principal Investigator or course director: Marilyn Scott SIGNATURE date: 11 (day) 03 (month) 03 (year)

Chairperson, Biohazards Committee: SIGNATURE date: 11 (day) 03 (month) 03 (year)

Approved period: beginning 11 (day) 03 (month) 03 (year) ending 31 (day) 03 (month) 07 (year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1998

(attach additional sheets if preferred)

| Name | Department | Check appropriate classification | | | | Fellow |
|---|---|---|---|---|---|---|
| | | Investigator | Technician & Research Assistant | Student | | |
| | | | | Undergraduate | Graduate | |
| Marilyn Scott | Institute of Parasitology | X | | | | |
| Cristina Gheorghiu | Institute of Parasitology | | | | X | |
| | | | | | | |
| | | | | | | |

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Gordon Bingham Phone No: work: 398-7731 home: 457-9138

Name: Cristina Gheorghiu Phone No: work: 398-8382 home: 457-7068

6. Briefly describe:

i) **the biohazardous material involved** (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

*Gyrodactylus turnbulli* is a monogenean ectoparasite of guppies. It is host specific and does not infect any local fish or any other animals including humans.

ii) **the procedures involving biohazards**

Lab-reared guppies are infected under anaesthetic with between 1 and 3 individual parasites attached to a scale or small piece of fin removed from another fish.

Infected fish are monitored daily under anaesthetic.

Survival of the parasite off the fish is limited a few hours at most. Water which has contained infected fish, as well as fish infected fish that have died are disposed of in biohazard containers.

iii) **the protocol for decontaminating spills**

The parasite is killed with a weak formalin solution, which will be used in case "contaminated" water or infected fish should come in contact with bench surfaces.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Not Applicable

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

Not Applicable

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10. List the biological safety cabinets to be used. Not relevant

| Building | Room No. | Manufacturer | Model No. | Serial No. | Date Certified |
|---|---|---|---|---|---|
| | | | | | |
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